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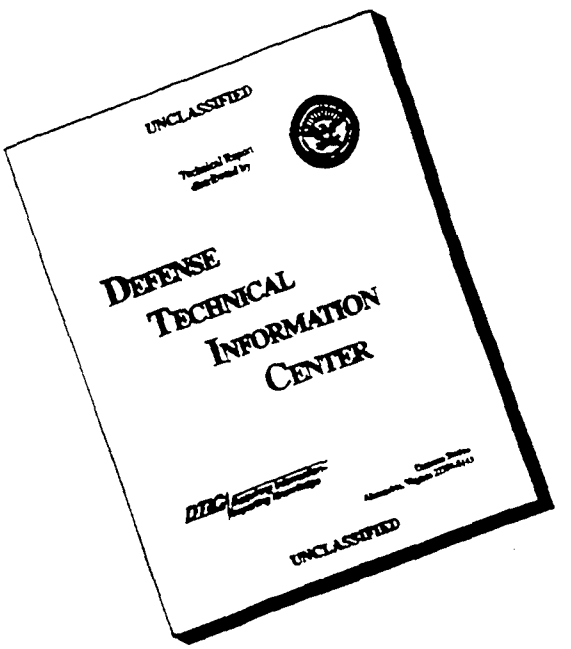

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Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that inhibits mammary carcinogenesis. Data obtained on this project indicate: 1) that the anticancer activity of CLA is unlikely to be explained based soley on its antioxidant activity; 2) that CLA is unlikely to inhibit carcinogenesis by directly antagonizing linoleic acid metabolism; 3) that CLA inhibits carcinogenesis irrespective of the presence or absence of the codon 61 mutation in the Ha-ras gene. Candidate genes that are over-expressed specifically in mammary carcinomas have been identified. The effects of CLA on the expression of these genes will be investigated to determined if they are involved in the inhibition of mammary carcinogenesis by CLA.

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Kenny Thomson 10/8/96

PI - Signature Date

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INTRODUCTION

PROPOSAL RELEVANCE. In 1981 Doll and Peto (1) estimated that nutrients and other dietary factors could account for a significant percentage of the risk for epithelial cancers in the United States and recently Doll (2) has suggested that approximately 35% of these cancers may be preventable via changes in dietary behaviors. Of the nutritional and dietary factors considered with regard to the risk for breast cancer, the role that the amount and type of dietary fat and calories play in the disease process has received prominent attention. This work has recently been reviewed (3,4). Two facts that have surfaced in this area of investigation are particularly relevant to the experiments being conducted. First, the level of caloric intake has a prominent effect on mammary tumorigenesis (3) and second, dietary fat has a specific effect on mammary tumorigenesis, but this effect is observed only when caloric intake is ad libitum (3). Our laboratory was one of the first to report the requirement for ad libitum intake for a fat specific effect on mammary tumorigenesis to be manifest (5), an observation that has recently been confirmed by others (6). It appears that this observation applies over a range of dietary fat concentrations. Given that a major health concern in the United States continues to be the consequences of intake of calories in excess of energy needs, it is probable that fat specific effects are being exerted in the U.S. population and other societies in which there is a surfeit of dietary calories.

As part of an overall public health initiative, Americans are being encouraged to eat less and exercise more in order to maintain "ideal" body weight, and to reduce the percent of dietary calories that they consume as fat (7). This advice is given with greatest specificity for prophylaxis of diseases of the heart, but these recommendations also apply to cancer, especially of the breast and colon. In general, it is recommended that dietary fat intake be reduced to $\leq$ 30% dietary calories with $\leq$ 10% provided as saturated fat, 10% as monounsaturated fat and 10% as polyunsaturated fat. An opportunity exists, therefore, to make recommendations about the specific fats that provide these calories. With regard to cancer, a principal interest lies in altering the type of polyunsaturated fatty acids (PUFA) that are being ingested. The question now receiving particular attention is whether all families of PUFA have similar effects on tumorigenesis and if individual fatty acids have selective effects on the mammary gland. ***The program of research being conducted on this grant specifically addresses this issue. We are investigating the cancer preventive activity of a specific fatty acid, conjugated linoleic acid (CLA), and we are studying various mechanisms that may account for its protective activity.***

CLA , a collective term that refers to conjugated dienoic derivatives of linoleic acid, is a naturally occurring substance in dairy products and in animal tissues. In a number of recent publications evidence has emerged indicating that CLA fed in the diet is a potent inhibitor of chemically-induced mammary carcinogenesis in the rat (8-11). This effect of CLA is in sharp contrast to that of linoleic acid which has been shown to stimulate the carcinogenic process in the same tumor model system in a dose dependent manner. Of added interest is the apparent potency of CLA in cancer prevention in comparison to other fatty acids reported to have cancer inhibitory activity. The most prominent among these are the fatty acids in fish oil. However, the amount of fish oil needed for cancer inhibitory activity usually exceeds 10% (w/w) in the diet. Recent work indicates that a level of CLA as low as 0.1% (w/w) was sufficient to produce a significant inhibition of mammary carcinogenesis. Thus, CLA is considerably more potent than any other fatty acid in inhibiting tumor development.

The potential relevance of these observations for cancer prevention in humans is considerable. In a direct extrapolation of the laboratory animal data to a 55 kg person, the amount of CLA required for cancer prevention would be equivalent to 2.8 g per day. The current estimate of

CLA consumption per day in the United States is 1 gram. The difference in these values is relatively small. Given that dietary levels of at least 1.5% CLA (w/w) can be fed chronically without adverse consequences, it appears that achieving a protective level of CLA consumption is quite feasible. CLA offers great potential as a preventive agent and could even be provided at effective levels via the food supply either via designer foods or as a dietary supplement.

In the work currently being conducted on this grant we are investigating the biological activity(s) of CLA that account for its cancer preventive activity. Our working hypothesis is that CLA affects the processes of clonal expansion and/or clonal selection via modulating genetic and/or epigenetic mechanisms obligatory for, or permissive to the carcinogenic process. This hypothesis is being evaluated by determining the effect of CLA on the expression of molecular markers relevant to the process of mammary carcinogenesis. These investigations may identify critical molecular events that can be targeted for cancer prevention.

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TECHNICAL OBJECTIVES OF THIS PROJECT

Objective 1. ***Does CLA inhibit the formation of oxidative damage to DNA?***

CLA has been reported to be a potent antioxidant in test tube assays, but its biological activity as an antioxidant is unclear. During the past year we have further investigated whether CLA has antioxidant activity in the mammary gland.

Objective 2. ***Does CLA alter the process of clonal expansion that occurs in the mammary gland in response to carcinogenic insult?***

During this reporting period it was determined the CLA inhibits mammary carcinogenesis irrespective of the presence of a mutation in codon 61 of the Ha-ras gene. Thus we undertook the identification of other pathogenetic markers in the model systems of mammary carcinogenesis that are being used. This gives us a new set of biomarkers by which to assess the effects of CLA on the process of clonal expansion.

Objective 3. ***Does CLA affect the process of clonal selection such that the pathogenetic pathway leading to mammary tumor formation is altered?***

The hypothesis that forms the basis for this objective is that CLA inhibits tumor occurrence by modulating the "activity" of specific genes, whose misregulation is central to the carcinogenic process. The key issue is to identify the genes that CLA modulates, and whether the effect is direct or indirect. CLA reduces but does not completely inhibit the occurrence of mammary carcinomas. We reason that treatment with CLA will result in the occurrence of a population of tumors that have a different spectrum of genetic defects than those tumors that occur in the absence of CLA treatment, i.e. that CLA will alter the process of clonal selection. The first set in achieving this objective has been accomplished in the identification of candidate genes that are over-expressed during the process of mammary carcinogenesis. Our goal is to determine whether the expression of these genes is affected in order to establish the causal basis for the cancer inhibitory activity of CLA.

BODY OF PROGRESS REPORT

The effort during the second year of funding was directed to continuing work on Technical Objective 1, and on work requisite to evaluation of the effects of CLA on clonal selection and clonal expansion (technical objectives 2 and 3. The following sections detail the methods that were developed to meet the goals stated in these objectives and the results obtained.

Materials and Methods

Source and composition of CLA and other dietary fats. The method of CLA synthesis from 99+% pure linoleic acid is detailed in reference (6, listed above). CLA was custom ordered from Nu-Chek, Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-,*t*9,*c*11- and *t*10,*c*12-CLA, constituted about 90% of the total. There were minimal variations in isomer distribution from batch to batch. Other fats used included: Mazola brand corn oil was obtained from Best Foods, Somerset, NJ, lard was purchased from Harlan Teklad, Madison, WI, menhaden oil was obtained from Marine Oil Test Program, U.S. Department of Interior, and palm oil was obtained from the Edible Oils Institute.

Animals and Diets

Animals. Female Sprague Dawley rats were used in the work reported. They were obtained from either Taconic Farms (Germantown, NY) or Charles River, Wilmington, Delaware. All rats were certified pathogen free.

Diet Formulations. A variety of diet formulations were used depending on the research question being addressed. All diets were modifications of the AIN-76A formulation and were designed to meet or exceed the known nutrient requirements of the rat unless otherwise specified.

Analytical Methods.

Analysis of urinary malondialdehyde (MDA). Following acid hydrolysis to release the bound form, MDA was derivatized with thiobarbituric acid (TBA) and the MDA-TBA adduct quantified by reverse phase HPLC with visible absorbance detection at 535nm. MDA content is expressed as nmol/mg creatinine.

In detail, 0.5 ml urine was combined with 5ul of an antioxidant solution containing 0.3M 2dp and 2% BHA in ethanol, and 40 ul concentrated HCL. The mixture was heated in a dry block at 96-99° for 4 and 3/4 hours. After samples had cooled slightly, 2 ml of TBA solution (1.11 % TBA in 74mM KOH) was added and the samples were heated at 96-99° for another 45 minutes. After cooling and immediately before HPLC analysis, samples were adjusted to a pH of 1.8 - 4.0 with 12N KOH. Previous method validation has confirmed that the presence of 2dp and BHA in urine samples during acid hydrolysis and TBA derivitzation prevents artifactual MDA contribution from food contamination in the urine, even with extreme contamination by menhaden oil containing diet. Creatinine was measured spectrophotometrically (Procedure 555, Sigma Diagnostics, St. Louis, MO 63178).

Determination of 8-OHdG and malondialdehyde in mammary tissue.

8-OHdG. For the assay of 8-OHdG, the various procedures of DNA purification from the mammary gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and dG by HPLC, and the quantitation of 8-OHdG with an electrochemical detector

were described in detail in a recent publication from our laboratory (11). Detector response was linear from 10 to >800 pg per injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues of 8-OHdG per 10^6 residues of dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Malondialdehyde (MDA). Tissue malondialdehyde was quantified as its thiobarbituric acid derivative with reverse phase HPLC and photometric absorbance detection at 535nm. In detail, mammary gland was homogenized with a Polytron in water containing 1% antioxidant solution (AOS: 0.3M dipyridyl and 2% BHA, in ethanol), 1 part mammary gland to 9 parts water (wt/vol). Homogenized samples were centrifuged at 6500 x g and fat plugs were removed, followed by further homogenization to re-suspend the pellet. As optimum reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.25 mg protein was prepared for hydrolysis. The homogenate was combined, in glass tubes, with 7.5 ul AOS and enough water to bring the volume to 1.47 ml. 7.5 ul 5N HCl was added, and covered tubes were heated to 96° C for 3 hours. Tubes were cooled quickly in tap water, and 30 ul sodium tungstate (Na_2WO_4) per tube was added to facilitate precipitation of protein. Tubes were centrifuged at 6500 x g for 10 min, and 1 ml of supernatant was then transferred from each to clean glass tubes. (The remaining supernatant and pellet were discarded.) 0.75 ml thiobarbituric acid (TBA) solution (1.11% TBA in 74 mM KOH) was added to each tube, and tubes were heated for 90 min for derivatization (to form TBA-MDA adduct). Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA-TBA adduct was separated using a 4.6 x 150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50mM potassium phosphate buffer, pH 6.0 delivered at 1.5 ml/min. Photometric absorbance detection was at 535nm. MDA was quantified by comparison of sample peak heights to those of standards, prepared from 1,1,3,3-tetramethoxypropane (TMP). To aliquots of stock standard were added water to 1.5 ml, 5 ul AOS, 1 ml TBA solution and 40 ul concentrated HCl. Standards were heated at 96° C for 14 min, cooled, and their pH adjusted to between 2.5 and 4.0 with 12N KOH. Final results were expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Determination of 8-OHdG concentration in liver DNA . The procedures described exhaustively herein contain significant changes from those previously described by us. The changes, such as eliminating phenol from the DNA isolation and adding BHT and 2-dp to buffers have been instrumental in reducing the contribution of artifacts to measured 8-OHdG. The importance of guarding against artifacts and their mistaken interpretation can not be over stated.

Isolation and enzymatic digestion of DNA from rat liver. DNA was isolated from liver with a phenol free process and was subsequently digested enzymatically to nucleosides for chromatographic analysis. In detail, 10ul of 26.4 mg/ml BHT was added to a 13 ml polypropylene screw cap tube, followed by 3 ml digestion buffer (100mM NaCl; 10mM Tris, pH8.0; 0.5% sodium dodecyl sulphate, pH 8.0, 400 ug/ml proteinase K (30 mAnson units/mg, cat # 24568, EC 3.4.21.14, from EM Science)) and approximately 75 mg frozen pulverized liver. The tube was inverted repeatedly to mix and incubated in a 50° water bath for16-20 hrs, after which it was removed from the bath and allowed to cool briefly before adding 1 ml 7.5M ammonium acetate and mixing thoroughly. The resulting precipitate was removed from suspension by centrifugation at 19000g for ten minutes at 4°, and the supernatant decanted and extracted twice with 24:1 chlroform/isoamyl alchohol. Nucleic acids were precipitated by the addition of 3 ml isopropanol, transferred to 1 ml silanized glass vials (Type I, Class A borosilicate glass, Waters Associates, Milford, MA) and the precipitate was washed with 70%

EtOH before dissolution in 340 ulTE buffer (10mM Tris; 1mM EDTA; pH 8.0) containing 5mM dp. RNA contamination was reduced by treating samples with RNase (55 ug in H_2O) for 1 hour at room temperature in the dark. After addition of 10 ul of 5M NaCl, DNA was precipitated by the addition of 350 ul isopropanol. While the presence of ribonucleosides does not interfere with the assay per se, removal of most of the RNA by treatment with RNase results in samples which are more readily digested to nucleosides and chromatographed. The DNA pellet was washed with 70% EtOH, dried briefly under reduced pressure without heat, and dissolved in100ul of 20mM sodium acetate, pH 4.8, containing 5mM DP. Dissolution was allowed to proceed overnight at room temperature in the dark prior to enzymatic digestion to nucleosides.

Chromatography of liver hydrolysate. 8-OHdG and dG were separated isocratically on a 4.6 X 250 mm Rainin Microsorb C18 column (5um, 100A) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was achieved on an ESA Coulochem Model 5100 A electrochemical detector equipped with a model 5011 analytical cell and a model 5020 guard cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported a residues 8-OHdG per million residues dG. The simultaneous analysis of both analytes from a single HPLC injection provided excellent precision without rigorously quantitative sample handling.

RNA isolation Total RNA was extracted from carcinomas and tissues by acidic phenol extraction using a commercial kit from BIOTECX Laboraotries, Inc. (Houston, TX). For differential display of mRNA, the total RNA preparations were digested with RNase-free DNase (GenHunter Corporation, Nashville, TN) in order to remove contaminating genomic DNA. For cDNA library construction, poly A(+) mRNA was enriched by oligo (dT)-cellulose column.

Differential display Differential display of mRNA was carried out with the RNAimage™ kit (GenHunter Corporation, Nashville TN) according to manufacturer's instructions with two minor modifications: 1) One tenth of the recommended amount of total RNA was used for the reverse transcription step in order to minimize an inhibitory activity(s) present in the mammary tissue RNA preparation; 2) It was found that an annealing temperature of 42 °C for PCR was optimal in Denver, CO to yield reproducible display patterns. Duplicate reactions were run for each primer combination. The PCR products (labeled by alpha-^{32}P-dATP) from the 3 mammary carcinomas (T_1, T_2 and T_3), the uninvolved mammary tissue and kidney were contrasted side-by-side on sequencing gels. Only those bands that were present in carcinoma lanes, but absent in mammary gland and kidney lanes were cut and re-amplified by PCR. The PCR products were size separated on low melting point agarose gel and band(s) of the expected size was eluted. The gel-purified PCR bands were used as templates to generate ^{32}P-labeled probes for Northern blot detection of gene expression on a screening panel of RNA preparations comprised of two kidney samples, two liver samples, the 3 mammary tumors that were used for the original differential display. In addition, the uninvolved mammary gland tissue and mammary gland tissue excised from a day-1 post-partum female rat were included in the screening panel. The RNA samples were size-separated by electrophoresis and transferred onto Nylon membrane for Northern blot detection of gene expression. GAPDH or cyclophylin gene was probed as an internal control for loading correction.

Cloning and Sequencing Those PCR bands that detected carcinoma-specific gene expression were cloned into pGEM-T vector (Promega, Madison, WI). For each band, four clones were inoculated and the plasmid DNA was isolated by an alkaline mini-prep procedure and at least two clones are sequenced on both strands by the dideoxy chain termination

method of Sanger (7) using a kit from US Biochemicals (St Louis, MO). A commercial service utilizing thermal cycle sequencing (Cornell DNA Service, Ithaca, NY) was also used to confirm the sequence of a few of the clones. The cloned gene fragments were used as templates to generate randomly labeled probes for Northern detection again to confirm that the cloned sequences corresponded to the genes originally detected by the PCR products from differential display gels. Sequence search was done using the BLASTN algorithm (8) with GenBank nr databases and expressed sequence databases dbEST.

Cloning full length cDNA A cDNA library was constructed with pooled poly(A)+ mRNA isolated from mammary carcinomas using the Marathon cDNA construction kit (Clontech, Inc, Palo Alto, CA). The average length of the library was 1.5 kb. Based on the sequence information obtained for each gene fragment, a gene-specific primer was synthesized (Integrated DNA Technology, Inc, Coralville, IA) as the down stream primer. A universal upstream primer that annealed to the adapter which had been ligated into the cDNA library and the gene-specific primer were used for long distance PCR using KlenTaq (a combination of Taq and Vent polymerases) to increase fidelity of cloning (ClonTech, Palo Alto, CA). The PCR fragments were cloned into the pGEM-T vector and sequenced as described above.

Ha-*ras* codon 12 mutation detection in mammary carcinomas The paraffin-embedded tumor blocks were cut into 5-*u*m sections. These sections were mounted on plastic slides coated with polylysine and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked for tissue retrieval. Pieces of a section (approximately 2x2 mm) were carefully cut with scissors from the marked area. The scissors were soaked in 10% Chlorox bleach and heat sterilized between samples to prevent carry-over. Each piece was incubated with proteinase K (400 :g/ml in 100 mM Tris-HCl, 2 mM EDTA) at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 5%-10% of the extract was used as the source of DNA for PCR amplification. This tissue collection procedure permits sampling of different regions of a carcinoma for PCR analyses. It is important to note that when this tissue sampling procedure was tested in independent experiments more than one hundred samples have been repeated at different times to check the reproducibility of the assay and to ensure the absence of carry-over and all of the repeated measures showed reproducible results.
The mutational status of Ha-*ras* codon 12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5'AGTGTGATTCTCATTGGCAG-3') was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene. The G → A mutation along with two mismatches in the downstream primer (5'-AGGGCACTCTTTCgaACGCC-3') introduces a XmnI restriction site into the PCR product (116 bp), which, upon digestion with the XmnI endonuclease of the PCR products, generates a fragment of 98 bp that is diagnostic for the mutation. A tracer amount of alpha-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography using Kodak X-ray films.

Results and Discussion

Appendix 1 contains four manuscripts, two have been submitted to Cancer Research, one to Free Radicals in Biology in Medicine and one to the journal Carcinogenesis. A reprint of one paper published in 1996 is also included. What follows are relevant excerpts from those manuscripts, and data that is currently being prepared for publication.

Evaluation of the Antioxidant Properties of Conjugated Linoleic Acid in the Rat. In order to further examine CLA's putative role as an *in vivo* antioxidant, we employed a model in which

rats were fed one of four experimental diets with either menhaden oil or palm oil as the predominant lipid and each formulated with or without CLA (1% w/w). The carcinogenic peroxisome proliferator clofibrate was added to all diets after initial 24 hour urine collections to provide an environment of high oxidative stress in which the sensitivity to detect antioxidant activity of CLA may be increased. Liver and mammary gland DNA were analyzed for 8-hydroxy-2'-deoxyguanosine content as an index of oxidative DNA damage. Malondialdehyde levels in urine, liver, and mammary gland were determined as indices of lipid peroxidation. In both the palm oil and menhaden oil diet groups mammary gland malondialdehyde content was lower in rats fed CLA than in rats fed the respective CLA-free diets. The difference was most striking in rats fed the highly peroxidizable menhaden oil diets ($p < .001$). However, the other indices measured failed to demonstrate antioxidant activity of CLA. The paucity of effect by dietary CLA on 8-OHdG levels in DNA from mammary gland and liver indicates that CLA does not modulate oxidative DNA damage. Liver and mammary gland MDA data indicate a tissue specific antioxidant function for CLA, possibly limited to neutral lipid stores. (Manuscript 1, Appendix)

Carcinoma clonality, multiplicity and anatomical regional differences of pathogenetic traits in 1-methyl-1-nitrosourea-induced rat mammary carcinogenesis. The induction of mammary carcinogenesis in the rat by 1-methyl-1-nitrosourea (MNU) is a widely used experimental model for breast cancer. In the experiments reported, the Ha-ras codon 12 mutation (GGA→GAA) was used as a molecular marker to address issues of the clonality of carcinomas induced, patho-genetic independence among multiple carcinomas within the same animal and distribution of this marker in carcinomas arising in different mammary gland chains. Carcinogenesis was induced in 50-day old female Sprague-Dawley rats by an intraperitoneal injection of MNU at a dose of either 25 or 37.5 mg MNU per kg body weight. In order to test the hypothesis that cancerous cells within a carcinoma share a common origin, 5-um sections of 44 MNU-induced mammary carcinomas were dissected, each resulting in 2 to 4 sampled sites per carcinoma and the Ha-ras codon 12 status in each site was analyzed by PCR-RFLP. Forty three carcinomas out of 44 (i.e., 97.7%) bore concordant Ha-ras genotypes among the multiple sites within each section, consistent with the monoclonal origin hypothesis. Upon histological analysis, the one carcinoma that showed discordant Ha-ras genotypes appeared to be composed of two separate carcinomas that had grown together. The relationship among multiple carcinomas within the same animal with respect to the occurrence of the Ha-ras mutation was examined. As carcinoma multiplicity per animal increased the observed discordance rate of Ha-ras genotypes increased in a manner that was consistent with the expected discordance rate based on assumption of no-association among carcinomas, indicative of independent initiation among multiple carcinomas within the same animal. Taken together, these data indicate that MNU-induced mammary carcinogenesis follows a monoclonal evolution of multiple, independently-initiated cells that emerge as distinct mammary carcinomas in the same animal. This information has two practical implications: 1) Representative sampling for genotying the Ha-ras marker can be achieved with high accuracy (~98%) with a single sample from a carcinoma section because of the monoclonal nature of carcinoma origin; and 2) the question of prevention or promotion of subpopulations of initiated cells can be studied because of the independent origin of multiple carcinomas within the same animal. Furthermore, a significant difference was observed in the occurrence of Ha-ras mutant carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands in that three times as many carcinomas were Ha-ras mutant in the former as in the latter glands, whereas the Ha-ras wild type carcinoma occurrence was approximately the same in both regions. This observation makes it imperative that all identifiable carcinomas be genotyped when hypotheses concerning effects of agents on pathogenetically distinct subpopulations of carcinomas are tested in this model. (Manuscript 2, Appendix).

Retention of Conjugated Linoleic Acid in the Mammary Gland is Associated With Tumor Inhibition During the Post-Initiation Phase of Carcinogenesis. CLA has been reported to have significant activity in inhibiting mammary carcinogenesis. A major objective of this study was to evaluate how the kinetics of CLA retention in mammary tissue as a function of CLA exposure/withdrawal was correlated with cancer prevention in this target organ. Rats treated with a single dose of DMBA at 50 days of age were given !% CLA in the diet for either 4 weeks, 8 weeks or continuously following carcinogen administration. No cancer protection was evident in the 4 or 8 week-CLA treatment groups. Significant tumor inhibition was observed only in rats that were given CLA for the entire duration of the experiment. Analysis of CLA in the mammary gland showed that the incorporation of CLA was much higher in neutral lipids than in phospholipids. When CLA was removed from the diet, neutral lipid- and phospholipid-CLA returned to basal values in about 4 and 8 weeks, respectively. The rate of decay of neutral lipid-CLA (rather than phospholipid-CLA) subsequent to CLA withdrawal paralleled more closely the rate of emergence of new tumors in the target tissue. It appears that neutral lipid-CLA may be a more sensitive marker of tumor protection than phospholipid-CLA. However, the physiological relevance of CLA accumulation in mammary lipids is unclear and remains to be determined. An additional goal of this study was to investigate whether CLA might selectively inhibit clonal expansion of DMBA-initiated mammary epithelial cells with wild type versus codon 61 mutated Ha-ras genes. Approximately 16% of carcinomas in the control group (no CLA) were found to express the codon 61 ras mutation. Although continuous treatment with CLA reduced the total number of carcinomas by 70%, it did not alter the proportion of ras mutant versus wild type carcinomas. Thus, based on analysis of the ras genotype marker, the data indicate the clonal selection process identified by this marker is not affected by CLA. (Manuscript 3, Appendix).

Gene expression changes associated with chemically-induced mammary carcinogenesis. Chemically-induced models of mammary carcinogenesis in the rat are widely used to study the biology of breast cancer development and to examine potential approaches for the prevention of this human disease. Whereas both genetic mutations and alterations in gene expression are being elucidated in the human disease, very little is known about gene expression changes that are associated with chemically-induced carcinogenesis in animal models. This paper reports the identification of over-expressed genes associated with mammary carcinogenesis induced by 1-methyl-1-nitrosourea using differential display of mRNA and molecular cloning. Nine over-expressed gene fragments were identified in mammary carcinomas in comparison with the uninvolved mammary gland, liver and kidney. Clone 3 represented the rat homologue of human galectin-7. Clone 4 was the homologue of the gene coding for the human melanoma inhibitory activity protein/bovine chondrocyte-derived retinoic acid sensitive protein (MIA/CD-RAP). Clone 16 and 18 were the homologues of the mouse/human DNA primase small unit and mouse stearyl-CoA desaturase-2 gene, respectively. Clones 10 and 14 were the homologue of mouse endo B cytokeratin/human cytokeratin-18 gene. Although these known genes have each been implicated in some aspect of carcinogenesis, their identification in this study is the first time that they have been associated with chemically induced mammary carcinogenesis. Clone 15 did not match any gene or expressed sequence tag (EST) whereas clones 1 and 9 showed significant homology to several ESTs. The expression patterns of clones 3, 4,10/14 and 18 were highly mammary carcinoma-specific when compared to 12 other normal tissues such as either virgin or lactating mammary gland, liver, heart and lung. The tumor-specific expression of these genes was also observed in rat mammary carcinomas induced by 7,12-dimethylbenz(alpha)anthracene (DMBA), indicating the generality of the role of the identified genes in rat mammary carcinogenesis. Full length cDNA has been cloned and sequenced for clone 3 and clone 4. Sequence comparison between cDNA obtained from rat fetal tissue RNA and that from mammary carcinoma RNA revealed no mutation in the MIA/CD-RAP coding region in the MNU-

induced mammary carcinomas. The gene expression changes identified in this study may represent useful molecular markers to understand mammary carcinogenesis in these model systems as well as in the human disease. (Manuscript 4, Appendix)

CONCLUSIONS

Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that has been shown to inhibit the development of experimentally-induced breast cancer. Data obtained on this project to date indicate: 1) that the anticancer activity of CLA is unlikely to explained based solely on its antioxidant activity in vivo, 2) that CLA is unlikely to inhibit mammary carcinogenesis by directly antagonizing linoleic acid metabolism, and 3) that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of the codon 61 mutation in the Ha-ras gene. During this reporting period candidate genes that are over-expressed specifically in mammary carcinomas were identified, and provide the basis for the further investigation of CLA's effects on genes that may be causally related to the development of mammary carcinomas.

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APPENDIX

MANUSCRIPT 1

EVALUATION OF THE ANTIOXIDANT PROPERTIES OF CONJUGATED LINOLEIC ACID IN THE RAT

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EVALUATION OF THE ANTIOXIDANT PROPERTIES OF CONJUGATED LINOLEIC ACID IN THE RAT

Abstract

Conjugated linoleic acid (CLA) has been reported to inhibit tumorigenesis in a number of animal models and to also inhibit lipid peroxidation. We examined CLA's antioxidant properties in rats fed one of two basal diets containing either menhaden oil or palm oil as the predominant lipid. Each was prepared both with and without CLA (1% w/w). The carcinogenic peroxisome proliferator, clofibrate, was added to all diets in order to provide an environment of high oxidative stress in which the antioxidant activity of CLA might be more readily observed. In both the palm oil and menhaden oil groups, mammary gland malondialdehyde (MDA) measurements were lower in rats fed CLA than in rats fed the respective CLA-free diets. The difference was striking in the menhaden oil diet groups ($p < .001$) and approached statistical significance in the palm oil diet groups ($p = .063$). However, 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in DNA from both liver and mammary gland, as well as urinary and liver MDA, were unaffected by dietary CLA supplementation. MDA and 8-OHdG content in liver were weakly but significantly correlated ($p=0.042$). CLA does not appear to inhibit carcinogenesis by protecting DNA from oxidative damage.

Key words - conjugated linoleic acid, oxidative DNA damage, lipid peroxidation, 8-hydroxy-2'-deoxyguanosine, malondialdehyde, peroxisome proliferator.

INTRODUCTION

Animal models in which conjugated linoleic acid (CLA) has been shown to inhibit carcinogenesis include methylnitrosourea (MNU)- and 7,12-dimethylbenz[*a*]anthracene (DMBA) -induced mammary cancer in the rat [1-4], benzo(*a*)pyrene (BP) -induced forestomach cancer in the mouse [5], and DMBA-induced, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) -promoted skin cancer in the mouse [6]. The mechanism of inhibition is not known but there is some evidence that CLA possesses both *in vivo* and *in vitro* antioxidant activity [1,4,5]. The role of oxidative stress in tumor development in the models mentioned above is unclear, making it difficult to delineate the relationship between the purported antioxidant property of CLA and cancer protection. In the study reported here, we sought to investigate both systemic and tissue specific antioxidant effects of CLA in rats that were exposed to different degrees of oxidative insult.

The oxidative status of the animals was modified by a combined dietary and pharmacologic approach. One of two basal diets was fed: a low peroxidation potential diet (LPX) containing predominantly palm oil and supplemented with adequate vitamin E, or a high peroxidation potential diet (HPX) containing predominantly menhaden oil and deficient in vitamin E. The LPX and HPX diets were formulated with and without CLA. The animals were also treated with clofibrate. Carcinogenic peroxisome proliferators such as clofibrate [2- (p-chlorophenoxy) -2-methylpropionic acid ethyl ester] are a novel class of hepatocarcinogens which do not react with DNA and exhibit no direct genotoxicity, either by the parent compound or a metabolically activated species [7,8]. A considerable body of evidence suggests that peroxisome proliferators induce carcinogenesis in rat liver via oxidative stress [7-10]. Thus, the addition of clofibrate to all diets in the present study was designed to intensify the oxidative climate in the liver, where oxidative damage is thought to be linked to carcinogenesis.

Urinary malondialdehyde (MDA) has been used extensively as an index of *in vivo* lipid peroxidation [11,12]. This marker was used here to assess the overall oxidative status of the animals before and after the administration of clofibrate. Because urinary MDA is indiscriminate of tissue origin, we also measured liver and mammary gland MDA. Oxidative DNA damage resulting in mutation is thought to be exacerbated by lipid peroxidation [13-15] and is a likely mechanism by which oxidative stress contributes to carcinogenesis [16-20]. Consequently, a decrease in oxidative DNA damage, as measured by 8-hydroxy-2'-deoxyguanosine (8-OHdG), could serve as a marker for the cancer protective effect of CLA. We therefore determined 8-OHdG in DNA extracted from liver, a target organ for clofibrate-induced carcinogenesis, and in DNA extracted from mammary gland, a tissue in which CLA has been shown to have potent cancer preventive activity [1-4].

MATERIALS AND METHODS

In evaluating the role of CLA as an antioxidant and the possible relationship of its antioxidant activity to carcinogenesis, methodological considerations are of critical importance. Thus the work presented in this paper focused considerable attention on experimental design and methodology.

Animals

Female Sprague Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 days of age. Rats were housed individually in metabolic cages in an environmentally controlled room maintained at 25° with 50% relative humidity and a 12 light-12 dark cycle. Distilled water was provided ad libitum.

Diet composition

Diet compositions are described in Tables I and II. Menhaden oil and corn oil were protected from oxidation with 0.02% tert-butylhydroquinone (TBHQ). This antioxidant is frequently used in dietary oils , but is minimally retained in animal tissues and is thought to possess little antioxidant activity *in vivo* [22,23]. Diets were stored frozen and were provided fresh every other day.

Menhaden oil was obtained from the Fish Oil Test Materials Program of the National Marine Fisheries Service. Corn oil containing no vitamin E was purchased from Eastman Kodak Company, Rochester, NY. Palm oil was obtained from Premier Edible Oils, Portland, OR. CLA was custom ordered from Nu-Chek, Elysian, MN. Clofibrate was purchased from Sigma, St. Louis, MO.

Experimental design

At 20 days of age rats were fed the LPX diet minus vitamin E for 21 days prior to being fed one of four experimental diets (HPX or LPX, with and without 1%CLA, n = 8-9 rats per group). Rats were fed the experimental diets for two weeks before urine collection began. MDA present in diets presents a potentially confounding problem, as a significant amount of ingested MDA is excreted in urine and is indistinguishable from that generated *in vivo*. Previous work in this laboratory (unpublished data) and that of others has shown that rats consuming diets containing menhaden oil can excrete large amounts of dietary MDA in urine, thereby allowing dietary MDA to masquerade as *in vivo* lipid peroxidation, if precautions are not taken [24]. Thus, for the night before the start of each urine collection and throughout the 24-hour collection period, rats were fed a "urine collection diet" (5% palm oil) thereby minimizing the contribution of ingested MDA to urinary MDA and equalizing that contribution among groups. Urine was

collected in a 24-hour period into vessels containing 5 mmol 2,2' -dipyridyl (DP) and 0.6 mmol butylated hydroxytoluene (BHT) in 10 ul methanol, plus 0.5 ml water. DP is an iron stabilizing reagent that has been shown to inhibit free radical producing reactions initiated by both FeII and FeIII [25]. BHT and DP were added to prevent oxidation of spilled food in the urine cup from contributing to measured MDA. After urine was collected three times at weekly intervals, all diets were modified to contain 0.125% (w/w) clofibrate. Rats were fed the clofibrate-containing diets for one week before urine was collected once weekly for three additional weeks. Following the final urine collection animals were killed, and livers and mammary glands excised and frozen promptly in liquid nitrogen.

Analytical methods

Oxidative damage indices are susceptible to artifacts induced by sample collection, preparation, and measurement. These artifacts are notoriously difficult to control and considerable vigilance must be exercised. The methods employed herein are therefore described in detail.

Urinary MDA determination

Following acid hydrolysis to release the bound forms, MDA was derivatized with thiobarbituric acid (TBA) and the MDA-TBA adduct quantified by reverse phase HPLC with spectrophotometric detection. Our method is a modification of that of Draper and Hadley [12]. In detail, 0.5 ml urine was combined with 5 ul of an antioxidant solution (AOS) containing 0.3M DP and 2% tert-butyl-4-hydroxyanisole (BHA) in ethanol, and 40 ul concentrated HCl. The mixture was heated in a dry block at 96-99° for 4 and 3/4 hours. This was followed by the addition of 2 ml of TBA solution (1.11 % TBA in 74 mM KOH) and heating

at 96-99° for another 45 minutes. The samples were quickly cooled by emersion in a room temperature water bath, and adjusted to a pH of 2.0 - 4.0 with 12 N KOH immediately before HPLC analysis. MDA was quantified by comparison of sample peak heights to a standard curve prepared with 1,1,3,3-tetramethoxypropane (TMP). TMP standards were simultaneously hydrolyzed to MDA and derivatized with TBA by treatment that differed from that of urine samples. To aliquots of stock standard were added water to 1.5 ml, 5 ul AOS, 1 ml TBA solution and 40 ul concentrated HCl. Standards were heated at 96° C for 14 min, cooled, and their pH adjusted to between 2.0 and 4.0 with 12N KOH. The MDA-TBA adduct was separated with a 4.6 x 150 mm Beckman Ultrasphere ODS C18 column and a mobile phase consisting of 32.5% methanol in 50 mM potassium phosphate buffer, pH 6.0, delivered at 1.5 ml/min. Photometric absorbance detection was at 535nm. The results are expressed as nmol/mg creatinine. Creatinine was measured spectrophotometrically with a commercial kit [26]. Previous method validation has confirmed that the presence of DP and BHA during acid hydrolysis and hydrolysis and TBA derivitization prevents the oxidation of lipids in food that may contaminate urine samples, thereby eliminating a source of MDA artifact.

Tissue MDA determination

Tissue MDA was quantified as its TBA derivative with reverse phase HPLC and photometric absorbance detection at 535nm. Our procedure is based on an extensive modification of the methods described in two publications [12,27]. We observed that measurement of tissue MDA was confounded by differences in the protein contents of tissue extracts that were hydrolysed and subsequently derivatized with TBA. By equalizing the protein concentration of the tissue extracts after homogenization, a significant problem of inconsistent MDA-TBA recovery from tissue extracts was resolved, and a potential source of error eliminated. To our knowledge this issue has not previously been reported in the assessment of tissue MDA.

In detail, mammary gland or liver was homogenized with a Polytron in water containing 1% AOS, 1 part tissue to 9 parts water (w/v). For mammary gland, homogenized samples were centrifuged at 6500 x g and fat plugs were removed , followed by further homogenization to re-suspend the pellet. As optimum reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.2 mg protein was prepared for hydrolysis. The homogenate was combined with 7.5 ul AOS, 7.5 ul 5N HCl, and enough water to bring the volume to 1.5 ml. The covered tubes were heated to 96° C for 3 hours, cooled quickly in tap water, and 30 ul sodium tungstate (Na_2WO_4) per tube was added to facilitate precipitation of protein. After centrifugation at 6500 x g for 10 min, 1 ml of supernatant was transferred to a clean glass tube. An aliquot of 0.75 ml TBA solution was added to each tube followed by heating for 90 min at 96° to form the MDA-TBA adduct. Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. Preparation of TMP standards and separation of the MDA-TBA adduct by HPLC were the same as described above. Results are expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent [28].

Determination of 8-OHdG concentration in isolated DNA from mammary gland and liver

DNA was isolated from tissue with a phenol-free process and was subsequently digested enzymatically to nucleosides for chromatographic analysis. The conditions described minimize the *ex vivo* formation of 8-OHdG. Frozen and pulverized tissue (200 mg mammary gland or 75 mg liver) was mixed with 10ul BHT (26.4 mg/ml) and 3 ml digestion buffer (100mM NaCl; 10mM Tris, pH8.0; 0.5% sodium dodecyl sulphate; 400 ug/ml proteinase K) in a polypropylene screwcap tube. The tube was incubated in a 50° water bath for 16-20 hrs, after which it was removed from the bath and allowed to cool briefly before the addition of 1 ml 7.5M ammonium acetate and thorough mixing. The resulting precipitate was removed from suspension by centrifugation at

19,000 g for ten minutes at 4°, and the supernatant decanted and extracted twice with 24:1 chloroform/isoamyl alcohol. Nucleic acids were precipitated by the addition of 3 ml isopropanol, and the precipitate was washed with 70% ethanol before dissolution in 340 ul TE buffer (10mM Tris; 1mM EDTA; pH 8.0) containing 5mM DP. RNA contamination was reduced by treating samples with RNase (55 ug in H_2O) for 1 hour at room temperature in the dark. After addition of 10 ul of 5M NaCl, DNA was precipitated by the addition of 350 ul isopropanol. While the presence of ribonucleosides does not interfere with the assay per se, removal of most of the RNA by treatment with RNase results in samples which are more readily digested to nucleosides and chromatographed. The DNA pellet was washed with 70% ethanol, dried briefly under reduced pressure without heat, and dissolved in 100ul of 20mM sodium acetate, pH 4.8, containing 5mM DP. Dissolution was allowed to proceed overnight at room temperature in the dark prior to enzymatic digestion to nucleosides, as previously described[15]. 8-OHdG and dG were separated isocratically on a 4.6 X 250 mm Rainin Microsorb C18 column (5um, 100A) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was achieved on an ESA Coulochem Model 5100 A electrochemical detector equipped with a model 5011 analytical cell and a model 5020 guard cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported as number of 8-OHdG per million dG. The simultaneous analysis of both 8-OHdG and dG from a single HPLC injection abrogated the need for a recovery standard or rigorously quantitative sample handling. 8-OHdG was generously provided by R.A. Floyd; dG was purchased from Boeringer Mannheim, Germany.

Data analysis and evaluation

All data were evaluated for distributional normality by probability plot analysis and were determined to be approximately normally distributed. These data were analyzed by analysis of variance and multivariate regression techniques. Statistical significance was established bt the use of two tailed probability distributions except when correlating tissue 8-OHdG and MDA, where one tailed tests were judged appropriate. The study design for assessing the effects of CLA on urinary MDA involved repeated measures of MDA in the same rats; this was accounted for in the statistical analysis of the data. Data presented in the figures are displayed with 95% confidence intervals about the means instead of the more customary standard error of the mean (SEM). This approach facilitates visual inspection of the statistical significance of differences among treatments, i.e. if the 95% confidence intervals of any given two groups overlap, they are not statistically different at $p < 0.05$ level, as evaluated by Student's two tailed t test[29].

RESULTS AND DISCUSSION

The results of urinary MDA measurement, summarized in Fig 1, indicate no antioxidant activity of CLA. To the contrary, mean urinary MDA tended to be higher when diets were supplemented with CLA, but the difference was not statistically significant ($p = 0.26$). A trend toward higher urinary MDA in HPX vs LPX diet groups that approached statistical significance ($p = 0.068$) was also observed. The impact of clofibrate as an oxidative stressor and the utility of urinary MDA as an oxidative index are evidenced by the striking difference in urinary MDA levels ($p < 0.001$) observed before and after clofibrate treatment. This marked increase in urinary MDA occurred within one week of adding clofibrate to the diets and continued during the following (and final) two weeks of the experiment.

The data in Fig. 2 show that lipid peroxidation in the mammary gland, as measured by MDA content, was markedly increased by the HPX compared to the LPX diet. The presence of

CLA in both diets reduced MDA levels in this tissue, an effect that was more pronounced with the HPX ($p < .001$) than the LPX ($p = 0.064$) diet. In contrast, the data in Fig. 3 show that liver MDA was not affected by exposure to CLA, even though the HPX diet resulted in more MDA than the LPX diet ($P<0.001$). The results of 8-OHdG analysis in DNA from mammary gland and liver are shown in Figure 4. No statistically significant differences among groups were noted, regardless of the type of diet or the presence or absence of CLA. Regression analyses of tissue 8-OHdG vs MDA in mammary gland and liver are shown in Figure 5. A positive association between liver 8-OHdG and liver MDA was observed ($p = 0.042$). A similar relationship was not observed in mammary gland.

The role of oxidants and antioxidants in the etiology and prevention of carcinogenesis continues to be a topic of interest and controversy. Part of the controversy in this field arises from the technical difficulty of estimating actual levels of oxidized macromolecules without artificially introducing their occurrence. Under carefully defined experimental conditions as described above, we tested the hypothesis that CLA acts as an antioxidant. First, we found that urinary MDA was a sensitive marker for detecting the large change in oxidative status known to be induced by feeding clofibrate, but changes in this marker that were attributed to feeding LPX versus HPX were small. This finding indicates that differences in systemic *in vivo* lipid peroxidation, as reflected by urinary MDA, that can be attributed to dietary conditions representing extremes of peroxidation potential is limited when potential confounding factors are controlled. With this in mind, we can say that the data reported provided no evidence that CLA has an overall antioxidant effect as measured by urinary MDA under conditions of low or high oxidative stress. However, the tissue data do show that CLA reduced MDA levels in the mammary gland, an effect that was magnified in the HPX compared to the LPX diet. This observation is consistent with the reported distribution of CLA in neutral lipid, the type of lipid that is predominant in the mammary gland. The lack of effect of CLA on liver MDA is also

consistent with the limited distribution of CLA reported to occur in phospholipid, which is proportionately much more abundant in liver than mammary gland. In a recent paper investigating antioxidant activity of CLA with an *in vitro* system, the presence of CLA did not protect membrane vesicles composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine from oxidative modification under various conditions [36]. Antioxidant activity for CLA with respect to membrane phospholipid is thus supported neither by *in vivo* data nor that from a model membrane system. These data indicate that CLA has *in vivo* antioxidant activity, but that it is likely limited to neutral lipid. The paucity of effect of CLA on 8-OHdG concentration in the mammary gland, as well as the apparent lack of a relationship between MDA and 8-OHdG in that tissue, suggest that CLA's anticancer activity is not due to protection of DNA from oxidative damage, despite the fact that CLA appears to be a potent inhibitor of both carcinogenesis and lipid peroxidation in the mammary gland, and a relationship between 8-OHdG and lipid peroxidation has been reported by others and has been inferred to have causal significance [8,34,35]. The failure of CLA to modulate liver MDA and 8-OHdG under conditions associated with the induction of hepatocarcinogenesis also argues against the likelihood that CLA's antioxidant activity accounts for its anticancer activity, especially since a positive association between 8-OHdG and MDA in the liver was observed (Figure 5B). It is possible that the antioxidant activity exhibited by CLA in the mammary gland mediates cancer protection by epigenetic mechanisms that are dissociated from the modulation of oxidative DNA damage. Collectively however, these data do not support the hypothesis that the antioxidant activity of CLA plays a prominent role in accounting for its anticancer activity.

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ABBREVIATIONS

CLA - conjugated linoleic acid

MDA - malondialdehyde

8-OHdG - 8-hydroxy-2'-deoxyguanosine

LPX - low peroxidation potential

HPX - high peroxidation potential

DP - 2,2'-dipyridyl

BHT - butylated hydroxytoluene

TBA - thiobarbituric acid

AOS - antioxidant solution

BHA - butylated hydroxyanisole

TMP - tetramethoxypropane

Table I. Diet Formulation

Ingredient[a]	Percent of Diet (w/w)
Solka floc	6.5
Corn starch	18.2
Cerelose	18.2
Casein	26.0
Vitamin mix[b] (vitamin E omitted)	1.30
Mineral mix[b]	4.55
DL-α-tocopherol acetate (LPX diets only)	0.006
DL-methionine	0.390
Choline bitartrate	0.260
Dietary oil of varied composition (see table II)	24.6

[a] Ingredients are of the grade specified [21].
[b]The AIN-76 formulations were used [21].

Table II. Dietary Oil Composition

Designated diet	Oil composition (w/w)
LPX	21.2 % palm oil, 3.4 % corn oil[a]
LPX+CLA	20.2 % palm oil, 3.4 % corn oil, 1.0 % CLA
HPX	17.9 % menhaden oil[a], 6.7 % corn oil
HPX+CLA	16.9 % menhaden oil, 6.7 % corn oil, 1.0 % CLA

[a] all corn and menhaden oil was free of vitamin E and preserved with 0.02 % TBHQ.

Fig. 1. Effects of diet type (HPX,LPX), CLA, and clofibrate on urinary MDA. Results of tests for fixed effects obtained with SAS proc mixed[a] model for the analysis of repeated measures data on urinary MDA in 34 rats are as follows:

Source	p value (two tailed t)
Diet type (HPX,LPX)	0.068
CLA status	0.257
Clofibrate status	<.001

[a] SAS/STAT Software Changes and Enhancements 6.11, SAS Institute, Cary, NC

Figure 1

Urinary MDA Levels by Treatment

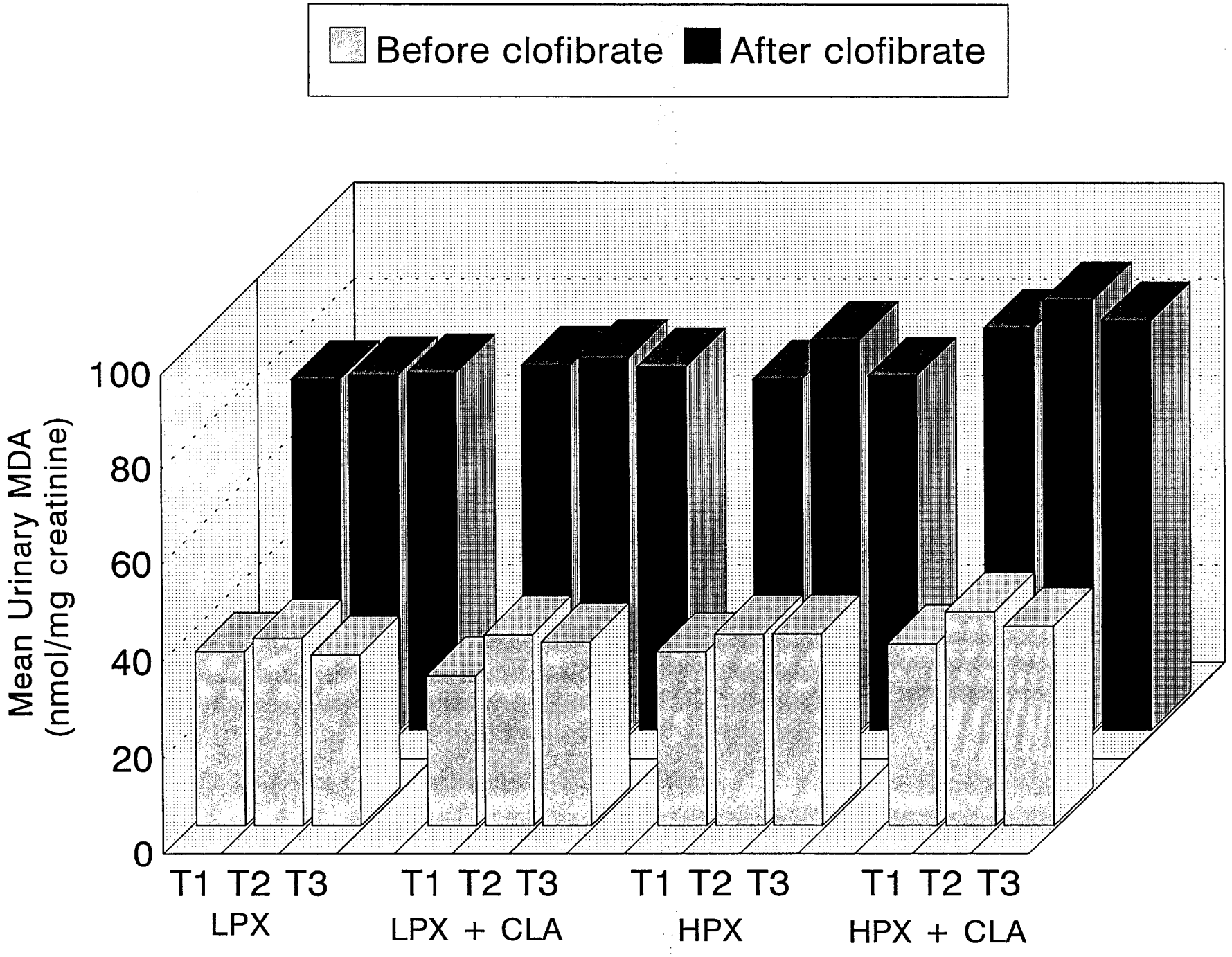


Fig. 2. Effects of diet treatment group on mammary gland MDA. n = 8-9 per group. Error bars represent 95% confidence intervals. Student's two tailed t test statistics for differences between groups are as follows:

Contrast	Difference of means	p value (two tailed t)
LPX vs LPX-CLA	.06	.064
HPX vs HPX-CLA	3.8	< .001
All LPX vs all HPX	3.1	< .001

Figure 2

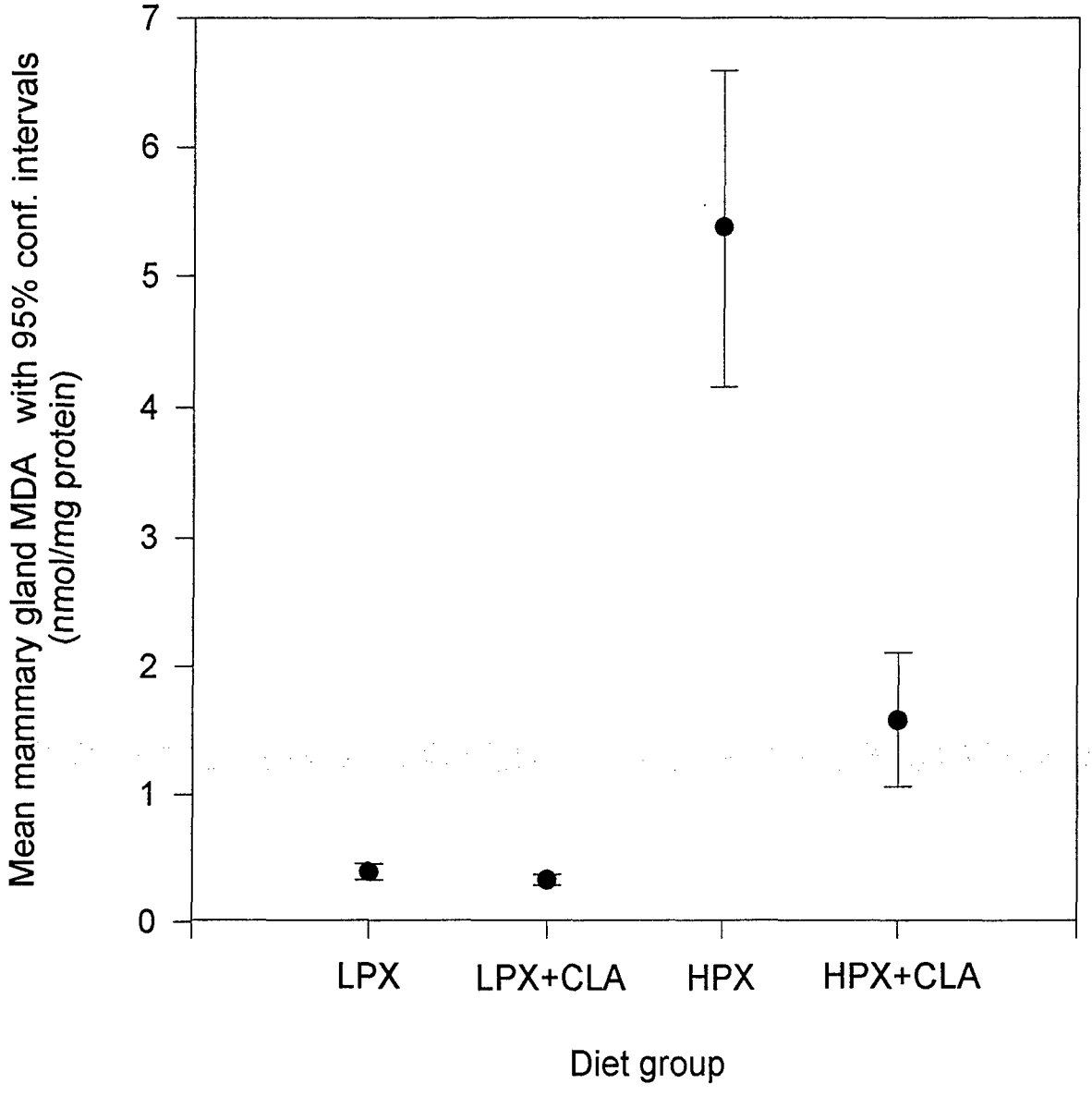

Mean mammary gland MDA with 95% conf. intervals
(nmol/mg protein)
7
6
5
4
3
2
1
0
LPX
LPX+CLA
HPX
HPX+CLA
Diet group

Fig. 3. Effects of diet treatment group on liver MDA. n = 8-9 per group. Error bars represent 95% confidence intervals. Student's two tailed t test statistics for differences between groups are as follows:

Contrast	Difference of means	p value (two tailed t)
LPX vs LPX-CLA	0.03	0.39
HPX vs HPX-CLA	0.01	0.83
All LPX vs all HPX	0.24	<.001

Figure 3

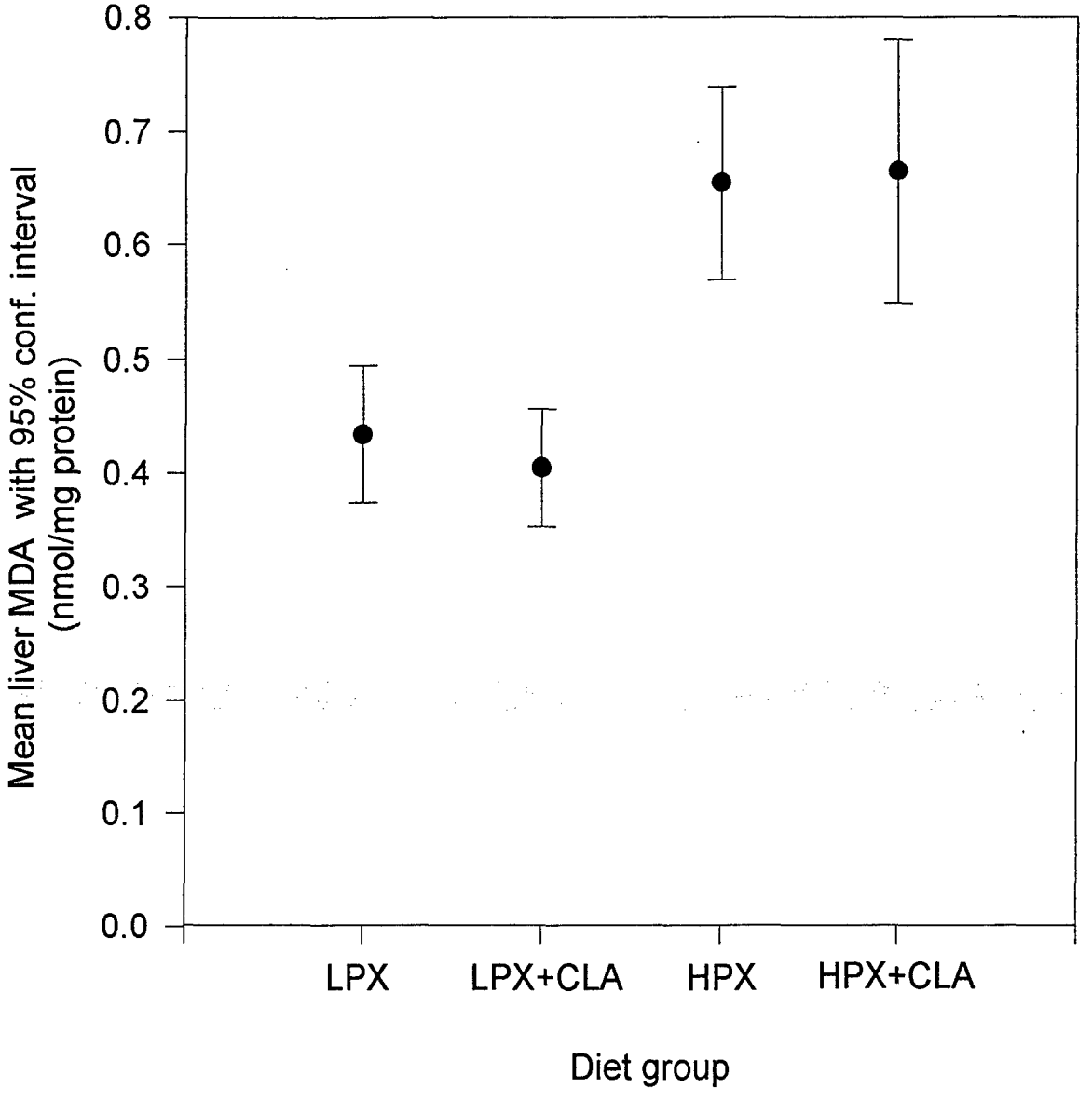

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Mean liver MDA with 95% conf. interval
(nmol/mg protein)
LPX
LPX+CLA
HPX
HPX+CLA
Diet group

Fig. 4. Effects of diet treatment group on 8-OHdG content of mammary gland (A) and liver (B). n = 8-9 per group. Error bars represent 95% confidence intervals. No significant differences between treatment groups were observed with Student's two tailed t test.

Figure 4A

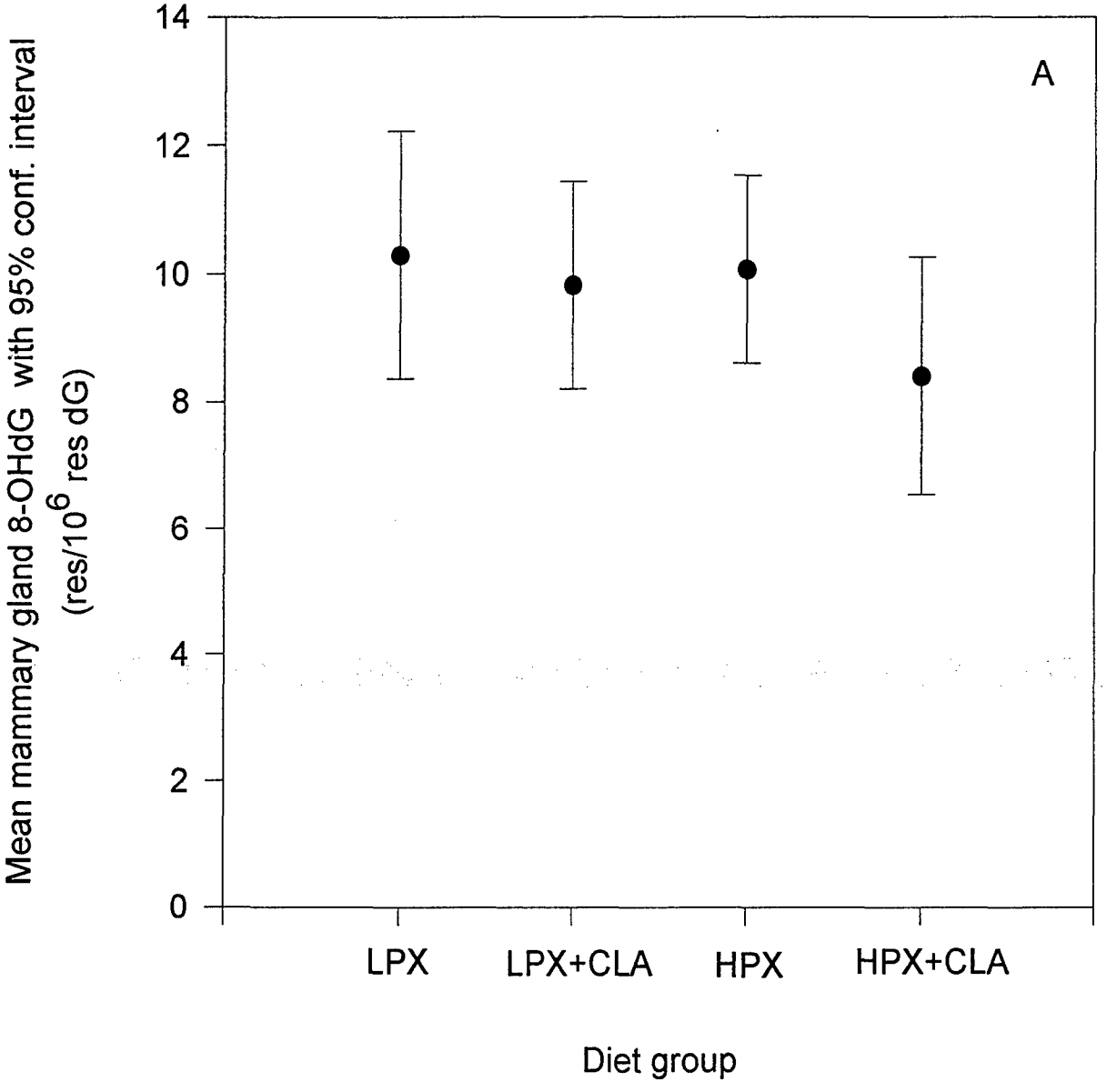

A
Mean mammary gland 8-OHdG with 95% conf. interval
(res/10^6 res dG)
14
12
10
8
6
4
2
0
LPX
LPX+CLA
HPX
HPX+CLA
Diet group

Figure 4B

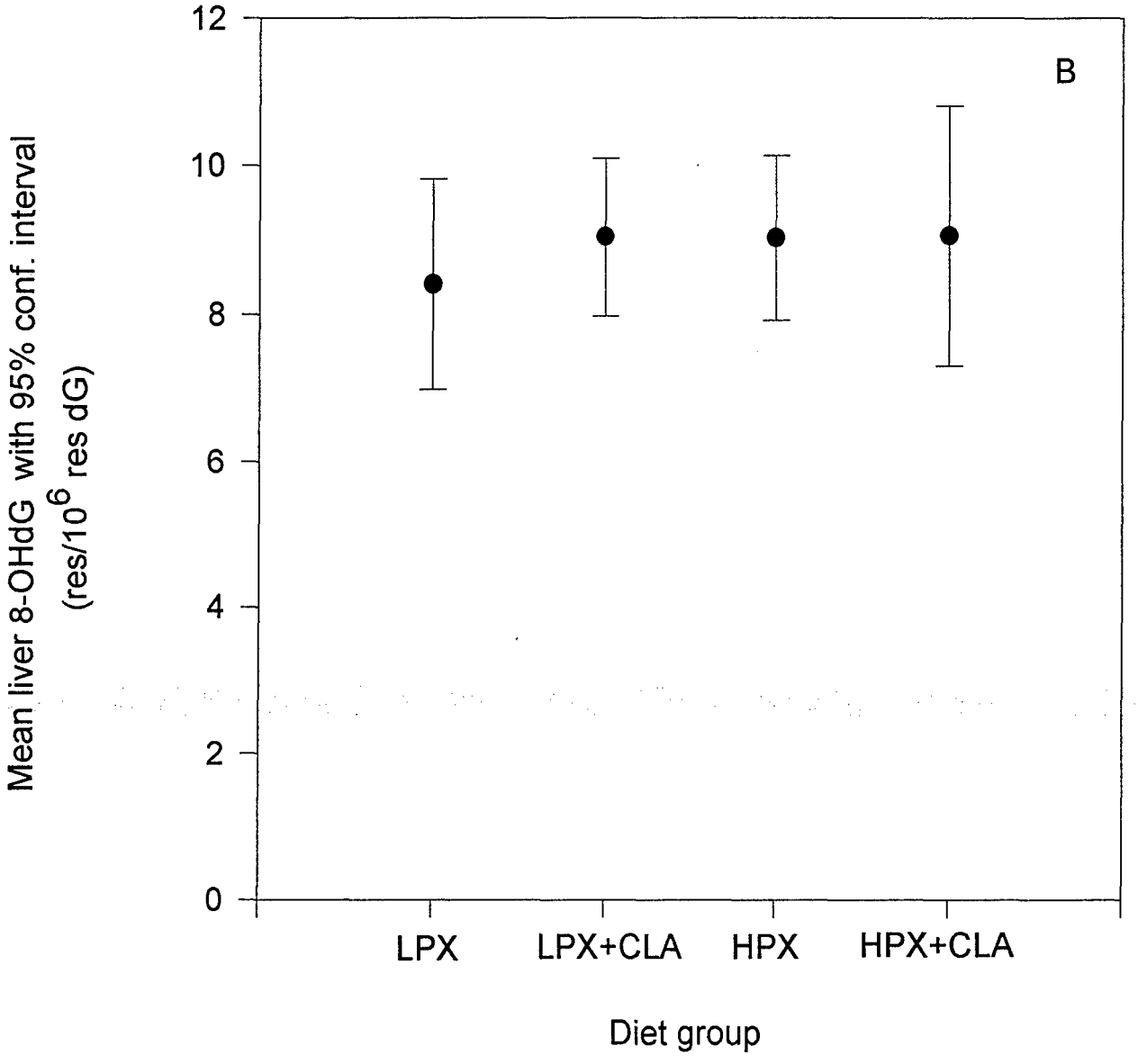

B
12
10
8
6
4
2
0
Mean liver 8-OHdG with 95% conf. interval
(res/10^6 res dG)
LPX
LPX+CLA
HPX
HPX+CLA
Diet group

Fig. 5. Regression analysis of tissue MDA and 8-OHdG in mammary gland (A) and liver (B). A small but significant correlation was observed in liver. n = 30; slope = 4.21; adjusted $r^2 = 0.071$; p (1 tail) = 0.042.

Figure 5A

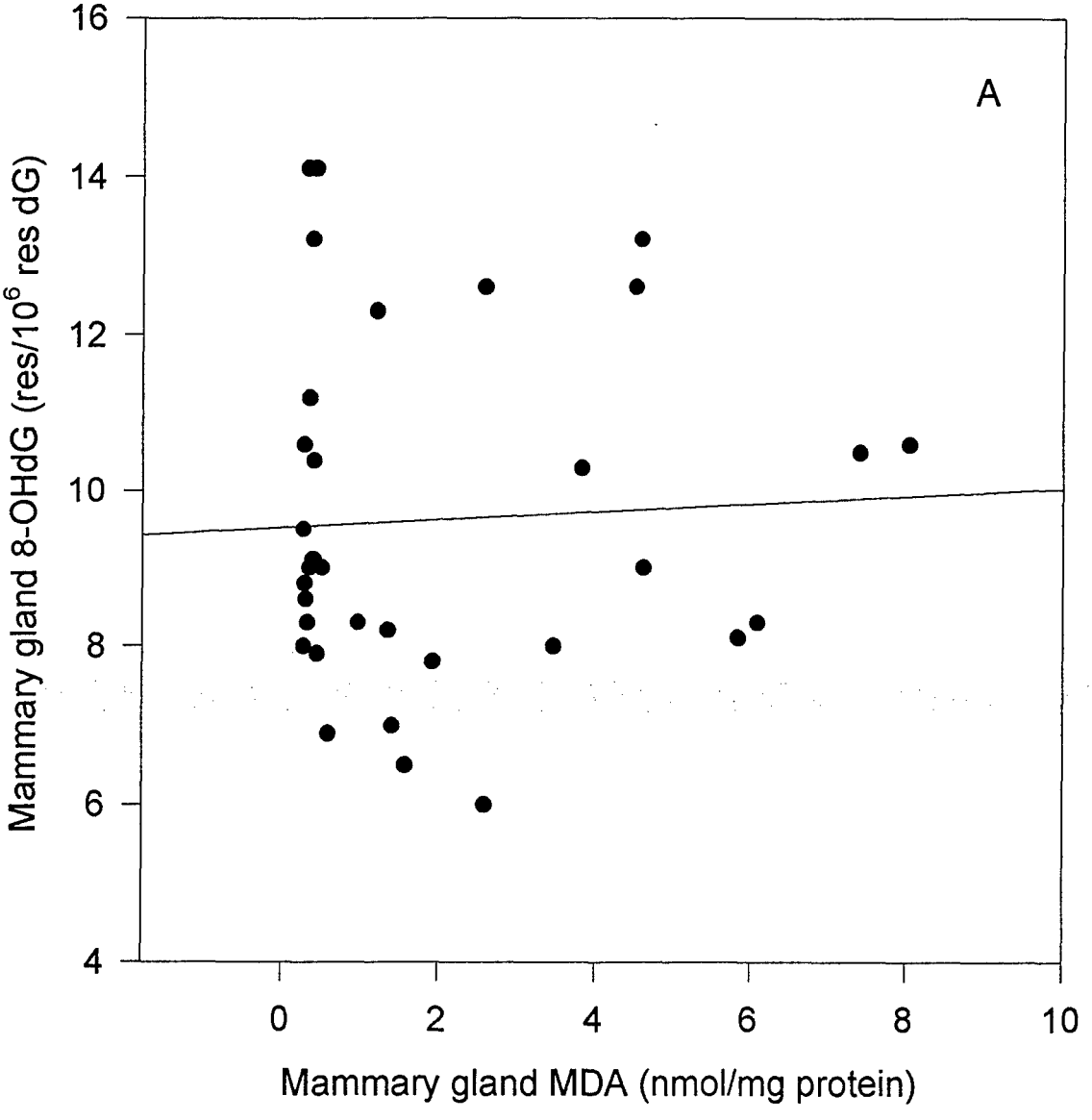
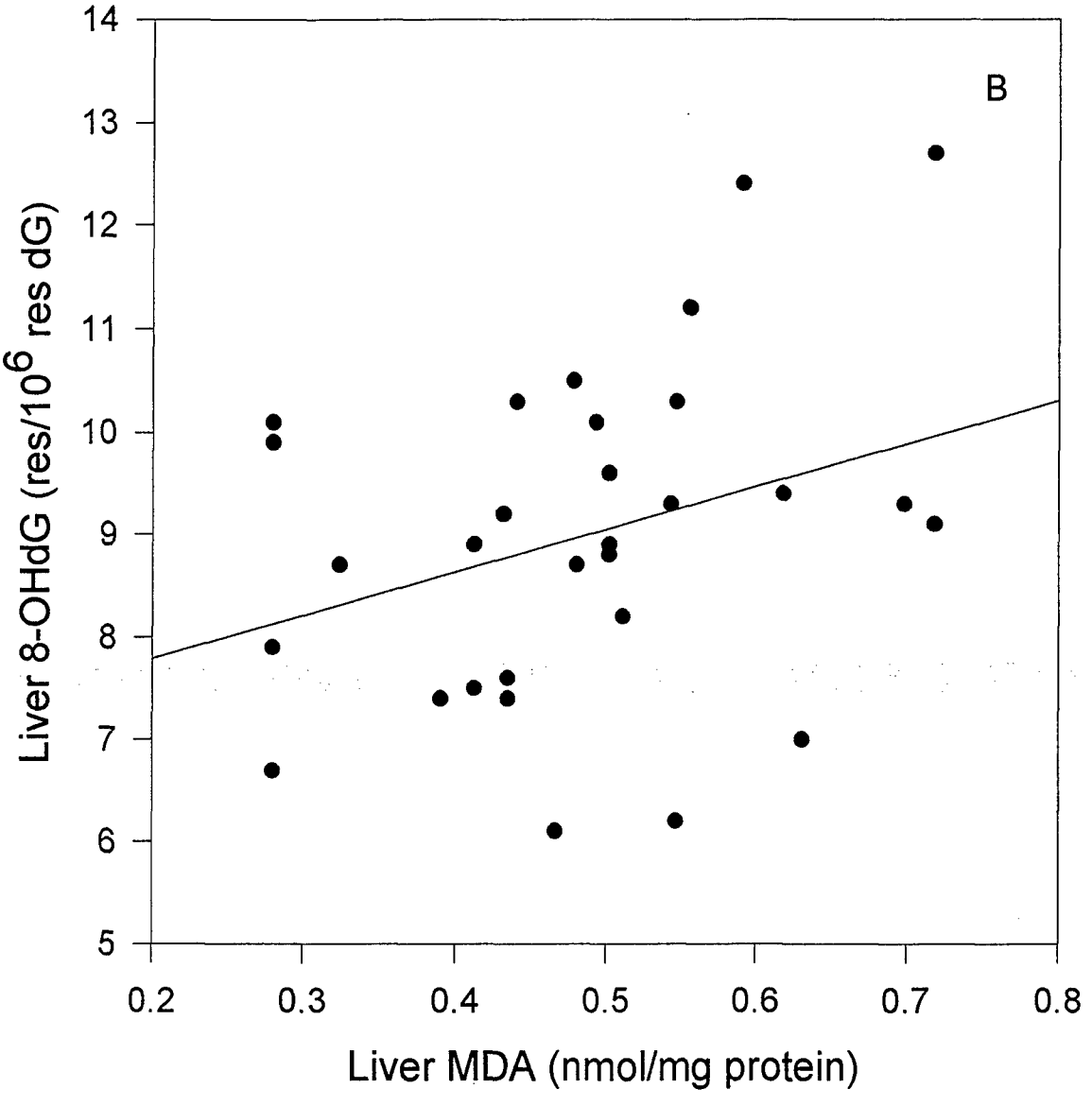

A
Mammary gland 8-OHdG (res/10^6 res dG)
16
14
12
10
8
6
4
0
2
4
6
8
10
Mammary gland MDA (nmol/mg protein)

Figure 5B


B
Liver 8-OHdG (res/10^6 res dG)
14
13
12
11
10
9
8
7
6
5
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Liver MDA (nmol/mg protein)

MANUSCRIPT 2

CARCINOMA CLONALITY, MULTIPLICITY AND ANATOMICAL REGIONAL DIFFERENCES OF PATHOGENETIC TRAITS IN 1-METHYL-1-NITROSOUREA-INDUCED RAT MAMMARY CARCINOGENESIS[1]

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Keywords: mammary carcinogenesis, carcinoma clonality, carcinoma multiplicity, 1-methyl-1-nitrosourea, Ha-ras oncogene

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ABSTRACT

The induction of mammary carcinogenesis in the rat by 1-methyl-1-nitrosourea (MNU) is a widely used experimental model for breast cancer. In the experiments reported, the Ha-ras codon 12 mutation (GGA→GAA) was used as a molecular marker to address issues of the clonality of carcinomas induced, patho-genetic independence among multiple carcinomas within the same animal and distribution of this marker in carcinomas arising in different mammary gland chains. Carcinogenesis was induced in 50-day old female Sprague-Dawley rats by an intraperitoneal injection of MNU at a dose of either 25 or 37.5 mg MNU per kg body weight. In order to test the hypothesis that cancerous cells within a carcinoma share a common origin, 5-um sections of 44 MNU-induced mammary carcinomas were dissected, each resulting in 2 to 4 sampled sites per carcinoma and the Ha-ras codon 12 status in each site was analyzed by PCR-RFLP. Forty three carcinomas out of 44 (i.e., 97.7%) bore concordant Ha-ras genotypes among the multiple sites within each section, consistent with the monoclonal origin hypothesis. Upon histological analysis, the one carcinoma that showed discordant Ha-ras genotypes appeared to be composed of two separate carcinomas that had grown together. The relationship among multiple carcinomas within the same animal with respect to the occurrence of the Ha-ras mutation was examined. As carcinoma multiplicity per animal increased the observed discordance rate of Ha-ras genotypes increased in a manner that was consistent with the expected discordance rate based on assumption of no-association among carcinomas, indicative of independent initiation among multiple carcinomas within the same animal. Taken together, these data indicate that MNU-induced mammary carcinogenesis follows a monoclonal evolution of multiple, independently-initiated cells that emerge as distinct mammary carcinomas in the same animal. This information has two practical implications: 1) Representative sampling for genotying the Ha-ras marker can be achieved with high accuracy

(~98%) with a single sample from a carcinoma section because of the monoclonal nature of carcinoma origin; and 2) the question of prevention or promotion of subpopulations of initiated cells can be studied because of the independent origin of multiple carcinomas within the same animal. Furthermore, a significant difference was observed in the occurrence of Ha-ras mutant carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands in that three times as many carcinomas were Ha-ras mutant in the former as in the latter glands, whereas the Ha-ras wild type carcinoma occurrence was approximately the same in both regions. This observation makes it imperative that all identifiable carcinomas be genotyped when hypotheses concerning effects of agents on pathogenetically distinct subpopulations of carcinomas are tested in this model.

INTRODUCTION

The 1-methyl-1-nitrosourea (MNU)-induced rat mammary carcinogenesis model (1) has contributed significantly to the current understanding of the biology of breast cancer and to potential approaches for its prevention. Major attributes of this model include that the proportion of mammary carcinomas that are ovarian dependent is similar to that observed in the human disease and that the carcinomas induced are aggressive and locally invasive (1). An additional advantage is that because MNU is a direct carcinogen, there is a clear distinction between the processes of cancer initiation and promotion in this model system. This latter feature of the model is often exploited to study effects of cancer preventive agents or risk factors on the promotion and progression stages of mammary carcinogenesis. Technical improvements since its original publication (1) have made this model easier to implement and more reproducible (2-4). For example, Thompson and coworkers (3, 4) have examined this model with respect to the route of carcinogen administration and have found that a single dose of MNU given intraperitoneally (i.p.) or subcutaneously (s.c.) was as effective as when it was given by intravenous (i.v.) injection, the method of administration originally reported (1,2). When MNU was administered by i.p. injection, smaller coefficients of variation in the number of carcinomas per rat were observed, an improvement the authors attributed to the consistent manner and the ease with which the MNU was delivered (4). The work reported here was based on the induction of mammary carcinogenesis by i.p. administration of MNU.

Experiments were designed to address the following issues: A) Significant morphological heterogeneity is consistently observed within mammary carcinomas, as has been described in detail (1), even though monoclonal origin of carcinoma cells is generally assumed. However,

whether such heterogeneity represents morphological progression of the progeny cells of a single initiated cell, or the fusion of distinct initiated cell populations has never been critically addressed in this system. Resolution of this issue would provide insight about comparability with the human disease for which recent evidence indicates a monoclonal origin based on X-chromosome inactivation as the molecular marker (5) in spite of the presence of considerable intratumor morphological heterogeneity. B) It is well established that the number of mammary carcinomas per rat increases with increasing MNU dose (3, 4, 6). Whether multiple carcinomas within the same animal share the same patho-genetic markers has not been critically evaluated. The underlying issue concerns the validity of statistical assumptions used in the evaluation of the carcinogenic response. C) Regional differences have been observed in the occurrence of mammary carcinomas in that the cervical-thoracic (C-T) mammary gland chains bear approximately twice as many carcinomas as do the abdominal-inguinal (A-I) mammary gland chains, irrespective of route or dose of MNU administration or the strain of rats used (1, 3, 4). However, little is known about the patho-genetic characteristics of the carcinomas in the different gland chains. With the help of the Ha-ras mutation as a molecular marker, we provide experimental data to address these questions.

The patho-genetic characteristics of this experimental model of breast cancer are being defined with the use of new molecular techniques. One of the identifiable somatic genetic changes is a G → A transition in codon 12 (GGA → GAA) of the Ha-*ras* proto-oncogene in a proportion of the carcinomas (7-9). In spite of a recent paper that has claimed that mammary epithelial cells bearing preexisting Ha-ras codon 12 mutation were merely promoted by the carcinogen (14), work of the last decade with this model convincingly shows that this mutation is an early event (7-11) probably as a result of methylation of the guanine nucleosides by MNU (12, 13). Because of the early nature of the Ha-ras mutation in MNU-

induced mammary carcinogenesis, the carcinomas and the initiated cells that give rise to them can therefore be classified into two patho-genetically distinct subpopulations, i.e., those with a mutant codon 12 Ha-ras and those with a wild type codon 12 Ha-ras gene. For brevity, WTras12 will designate a genotype that is wild type at codon 12 (i.e., GGA) and mras12 the mutant at codon 12 (i.e., GAA).

MATERIALS AND METHODS

Animals, diets and carcinogenesis Female Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY) at 21 days of age and fed a modified AIN76A diet until 50 days of age. At 50 days, they were injected intraperitoneally with MNU by the method reported by Thompson and Adlakha (4). The dose level was 37.5 and 25 mg MNU per kg body weight (BW) for experiments 1 and 2, respectively. The rats were palpated for mammary tumors twice per week. When a tumor was first palpated, the date and the tumor location were recorded. The experiments were terminated at 22 and 25 weeks post-carcinogen for experiments 1 and 2, respectively. At necropsy, tumors and suspicious lesions were excised, fixed in 10% neutral buffered formalin (12 h) and later embedded in paraffin and sectioned for histological evaluation. The histopathological criteria were as described by Young and Hallowes (15). Only tumors that were classified as carcinomas were used for genotyping the Ha-*ras* codon 12 status.

Ha-*ras* codon 12 mutation detection in mammary carcinomas The paraffin-embedded tumor blocks were cut into 5-*u*m sections. These sections were mounted on plastic slides coated with polylysine and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked for tissue retrieval. Pieces of a section (approximately 2x2 mm) were carefully cut with scissors from the marked area. The scissors

were soaked in 10% Chlorox bleach and heat sterilized between samples to prevent carry-over. Each piece was incubated with proteinase K (400 µg/ml in 100 mM Tris-HCl, 2 mM EDTA) at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 5%-10% of the extract was used as the source of DNA for PCR amplification. This tissue collection procedure permits sampling of different regions of a carcinoma for PCR analyses. It is important to note that when this tissue sampling procedure was tested in independent experiments more than one hundred samples have been repeated at different times to check the reproducibility of the assay and to ensure the absence of carry-over and all of the repeated measures showed reproducible results.

The mutational status of Ha-*ras* codon 12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5'AGTGTGATTCTCATTGGCAG-3') was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene. The G → A mutation along with two mismatches in the downstream primer (5'-AGGGCACTCTTTCgaACGCC-3') introduces a XmnI restriction site into the PCR product (116 bp), which, upon digestion with the XmnI endonuclease of the PCR products, generates a fragment of 98 bp that is diagnostic for the mutation. A tracer amount of alpha-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography using Kodak X-ray films. A representative gel is shown in Figure 1C.

Statistical methods used in the analyses of these experiments included descriptive statistics, chi-square tests including Mantel-Haenszel tests for homogeneity of the association stratified by number of tumors per animal.

RESULTS

1. Clonality of cancerous cells within a carcinoma

Our interest in the monoclonal or polyclonal origin of chemically induced mammary carcinogenesis was prompted by the observation that the gross anatomy of mammary carcinomas revealed that they were usually composed of distinct lobes (examples are shown in Figure 1A). Tumor Rat 235 T5 displayed two portions that stained very differently by hematoxylin and eosin (H&E). When it was assessed by PCR-RFLP analyses of the Ha-ras genotype, one portion was WTras12 and the other mras12 (Figure 1C). This observation raised the possibility that this carcinoma was composed of two patho-genetically distinct carcinomas that had grown together during their development. Therefore, we decided to determine how common it was to observe different areas or lobes within a carcinoma to be composed of different subpopulations of cells as marked by presence of mras12 vs. WTras12. The hypothesis was that rather than being polyclonal in origin (see Scheme 1, Figure 1B), the majority of the mammary carcinomas induced in this model resulted from morphological diversification of progeny cells derived from a single initiated cell, i.e., monoclonal origin (See Scheme 2, Figure 1B).

To test this hypothesis, we randomly selected 43 additional carcinomas and carefully dissected tissue (to prevent carry-over) from multiple sites of any given section. In total, 24 carcinomas were sampled with 2 sites each, 5 carcinomas with 3 sites each and 14 carcinomas with 4 sites each. Each of the sampled sites was genotyped for Ha-ras codon 12 as shown in Figure 1C. All 43 mammary carcinomas analyzed showed concordant Ha-ras status (i.e., either all sites were mutant or all sites were wild type) among the multiple sites sampled (Table 1). Therefore, these data support a monoclonal origin for the separate lobes within a carcinoma based on the Ha-ras as a marker .

2. The pathogenetic profile of multiple carcinomas within the same animal

Do multiple carcinomas within the same animal share the same patho-genetic characteristics? If the answer to this questions is yes, it should follow that multiple carcinomas within an animal will not display discordant Ha-ras genotype (i.e., at least one carcinoma is different from the rest) because all carcinomas are either mutant ras or wild type ras. As shown in Figure 1C and tabulated in Table 2, the data did not support this hypothesis. Instead, the observed discordance rates were consistent with the probabilities predicted based on independent origin among carcinomas (as illustrated in Figure 1B, Scheme 1). The result was observed in two independent experiments in which different amounts of carcinogen were used to induce mammary carcinogenesis.

3. Disproportional distribution of mutant Ha-ras carcinomas in different anatomical regions

As shown in Table 3, significant regional differences between the cervical-thoracic (C-T) mammary gland chains and the abdominal-inguinal (A-I) mammary gland chains in the number of carcinomas induced were observed in both experiments. There were approximately twice as many carcinomas in the C-T glands as in the A-I glands, confirming earlier reports of anatomical regional difference for carcinoma occurrence in this model (1, 3, 4). However, a surprising finding was the disproportional distribution of mutant ras carcinomas between the C-T glands and the A-I glands (Table 3). Specifically, more than 3 times as many mutant ras carcinomas were located in the C-T glands as in the A-I glands, whereas the wild type ras carcinomas were almost equally distributed between the two regions (Table 3). The overall disproportional distribution pattern held true upon secondary analyses stratifying by the number of carcinomas per animal and by experiment. Relevant to the question of the overall Ha-ras mutation frequency in this model, Zhang and Gould have reported that the proportion of Ha-ras

mutant carcinomas is inversely correlated to the dose of MNU (10). That conclusion was supported by a similar inverse relationship between MNU dose and Ha-ras mutation proportion (Table 3, compare overall Ha-ras mutation rate in Experiments 1 and 2).

DISCUSSION

The objective of this paper was to address three issues: the clonality of cells within MNU-induced mammary carcinomas; the independence of occurrence of multiple carcinomas in the same animal and whether distribution of mammary carcinomas classified by Ha-ras as a patho-genetic marker was similar among mammary gland chains. The experimental data presented support the monoclonal origin of individual carcinomas based on the Ha-ras codon 12 GGA → GAA mutation as the molecular marker, in spite of the often-observed distinct lobes within a carcinoma. Morphological heterogeneity within carcinomas is therefore likely the result of diversification of progeny cells of the original initiated cell during clonal expansion and subsequent selection as carcinomas develop. Aside from its importance in revealing the clonal expansion aspect of the cancer biology in this model, this information is also valuable from a practical point of view as it indicates that representative sampling of tissue for genotyping purposes, at least as far as the Ha-ras mutation is concerned, can be achieved by sampling a single site with an accuracy of ~98%.

With respect to multiple carcinomas occurring in the same animal, the data showed that their Ha-ras codon 12 genotypes were independent of one another, that is, the ras genotype of one carcinoma in an animal does not predict that of the others in the same animal. The data were consistent with the hypothesis that multiple independently initiated epithelial cells upon carcinogen exposure were likely to give rise to the multiple carcinomas analyzed. The independent nature of individual carcinomas within an animal supports the use of carcinoma

multiplicity as a parameter for assessing the effects of preventive agents as well as risk factors. Taken together, the data discussed above support the notion that MNU induces initiation in multiple, independent epithelial cells each of which undergoes monoclonal expansion during ensuing promotion and progression to give rise to distinct carcinomas. It should be noted, however, that the independent nature of initiation inferred here is true only at the molecular marker level. Our data do not rule out physiological (i.e., epigenetic) interdependence among carcinomas within the same animal. Such an epigenetic interaction among carcinomas or initiated cells can potentially result from changes in the endocrine factors and metabolic milieu brought about by a preexisting carcinoma and could influence the emergence of additional carcinomas in the same animal and/or the latency of their appearance. In an early study, the kinetics of carcinoma appearance was observed to slow down significantly after the appearance of the first tumor (2). The implication of a secreted inhibitory factor from a primary tumor in suppressing the emergence of secondary tumors (18) highlights the complexity of this issue.

A surprising observation reported in this paper was the significant regional differences in the occurrence of mutant Ha-ras carcinomas in the C-T mammary glands and the A-I mammary glands. In fact, the previously observed 2:1 C-T to A-I ratio of carcinoma occurrence (1,3,4) could be almost entirely attributed to this preferential localization of mutant ras carcinomas in the C-T mammary gland chains. Whether the predominance of Ha-ras mutation in the C-T carcinomas reflects a preferential mutagenesis by MNU in favor of the C-T mammary glands or a differential regional expression of the mutant ras gene product which in turn may confer a selection advantage to those initiated cells with the mutation remains to be determined. Nonetheless, the practical implication of the observed regional differences should not be overlooked. Until the cause and the biological significance of the regional differences observed

in this study are clearly understood, it is advisable to follow a consistent tissue collection protocol with respect to carcinoma location in the mammary gland chains so that this source of bias is minimized when carcinoma tissues are collected for biochemical and cytological assessment.

The sampling issue is especially significant when "gene/pathway-specific" prevention of subpopulations of pathogenetically identifiable neoplasia is concerned. We and others have published experimental data that were consistent with selective promotion (16,19) or inhibition (20) of subpopulations of initiated cells based on either Ha-ras or Ki-ras mutation as markers. For such applications of molecular markers, it is imperative that all identifiable lesions be genotyped.

In summary, experimental data presented in this study support monoclonal evolution of multiple, independently-initiated cells that give rise to distinct mammary carcinomas in the same animal. This information has two practical implications: 1) Representative sampling for genotying the Ha-ras marker can be achieved with high accuracy (98%) with a single sample from a tumor because of the monoclonal nature of tumor origin; and 2) the question of prevention of subpopulations of initiated cells can be studied with molecular markers because of the independent origin of the multiple carcinomas within the same animal. A significant difference was observed in the occurrence of Ha-ras mutant carcinomas between the C-T and the A-I mammary glands. This observation makes it imperative that all identifiable carcinomas be genotyped when hypotheses concerning effects of agents on pathogenetically distinct subpopulations of carcinomas are tested in this model.

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Figure legend

Figure 1. A. Examples of the gross appearance of tumor sections (H&E) on thin plastic slides. The horizontal bar represents 1 cm in length. Sample code: Rat234T6 -- Rat #234, tumor #6. Lower case letters indicate sites from which tissue was sampled for Ha-ras genotyping. B. Schematic illustration of multiple independent origins (polyclonality, Scheme 1) and a common origin of cancerous cells within a carcinoma (monoclonality, Scheme 2). C. PCR-RFLP analysis of Ha-ras codon 12 genotype in carcinomas. P, positive control for codon 12 mutation. N, negative control for ras mutation, i.e., untreated rat mammary gland DNA (wild type ras codon 12). The presence of the shorter band (98 bp) is diagnostic of the ras codon 12 mutation.

Table 1. Ha-ras codon 12 genotyping of multiple sites of randomly selected mammary carcinomas from Experiment 1

Number of sites analyzed per tumor (n)	Predicted ras concordance rate among sampled sites		Observed number of carcinomas with		Observed ras concordance rate among sampled sites
	Assuming polyclonality[1]	Assuming monoclonality[2]	concordant ras among sampled sites	discordant ras among sampled sites	
2	0.505	1	24	1[3]	0.96
3	0.258	1	5	0	1
4	0.132	1	14	0	1
Total			43	1	0.98

[1]Predicted concordance rate based on multiple, independent origins for cells in different lobes within a carcinoma (See figure 1B, Scheme 1). The probability by chance for, say 3 sites, to show the same ras mutant genotype is pxpxp and to show wild type ras genotype is qxqxq. Thus the overall concordance probability = p^3+q^3 . For n sites sampled, the predicted concordance is calculated by formula $p^n + q^n$, where p = probability for mutant ras12 and q = 1-p = probability for WTras12 in a given site. p was estimated by the overall frequency of mutant ras carcinomas and in this experiment, p=0.45.

[2]Predicted based on monoclonal origin. The discordant Ha-ras genotypes among different sites is 0 because all sites will be either WTras12 or mras12. The concordant rate is independent of the number of sites (n) sampled.

[3]This section (Rat 235 Tumor 5) displayed two distinctly H&E-stained regions. The discordant Ha-ras genenotypes of the two portions sampled indicated that this section represented two independently initiated tumors growing together side-by-side.

Table 2. Ha-ras genotype profile of multiple mammary carcinomas within the same animals

Number of carcinomas borne by a rat (n)	Predicted ras discordance rate among multiple tumors assuming independent origin[1]	Number of rats with concordant ras genotypes among tumors	Number of rats with discordant ras genotypes among tumors	Total number of rats in category	Observed ras discordance rate among multiple tumors
Experiment 1 37.5 mg MNU/kg					
2	0.495	11	10	21	0.476
3	0.742	3	8	11	0.727
4	0.868	0	8	8	1
5	0.931	0	11	11	1
6	0.964	1	9	10	0.9
7 or greater	>0.981	0	17	17	1
Experiment 2 25 mg MNU/kg					
2	0.442	22	22	44	0.5
3	0.663	8	12	20	0.6
4	0.787	4	11	15	0.73
5	0.861	2	4	6	0.67
6 or greater	>0.908	0	3	3	1

[1]Predicted discordance rate among multiple tumors borne by the same animal assuming independence among tumors. Calculated by formula $1-(p^n+q^n)$, where p was estimated by the overall ras mutation frequency in carcinomas. $p=0.45$, $q=1-p=0.55$ in Experiment 1 and $p=0.67$, $q=0.33$ in Experiment 2, respectively. $n=$ number of carcinomas per rat.

Table 3 Distribution of mutant and wild type Ha-ras mammary carcinomas by anatomical regions

Location of glands	Number of carcinomas with		Sub Total	% Ha-ras mutation	X^2, p value[1]
	mutant ras	wild type ras			
Experiment 1 (37.5 mg MNU per kg)					
Cervical-Thoracic chains	132	115	247	53%	
Abdominal-Inguinal chains	41	91	132	31%	
Subtotal	173	206	379	45%	17.4 ($p<0.005$)
Experiment 2 (25 mg MNU per kg)					
Cervical-Thoracic chains	171	61	232	74%	
Abdominal-Inguinal chains	54	51	105	51%	
Subtotal	225	112	337	67%	16.1 ($p<0.005$)

[1]2x2 contingency table. Degree of freedom = 1. The strong association between the anatomical region and occurrence of ras mutation carcinomas observed in both experiments were further examined by stratifying over the total number of tumors per animal, and by experiment. The overall Cochran-Mantel-Haenszel $X^2 = 33$, $p<0.001$. The disproportional pattern of mutant ras carcinoma occurrence was observed for each of the 10 strata in Experiment 1 and 7 out of 8 strata in Experiment 2. The probability for such observed disproportional distribution occurring by chance is $p < 0.01$. This secondary analyses did not support the existence of bias of the distribution pattern due to tumor multiplicity per animal.

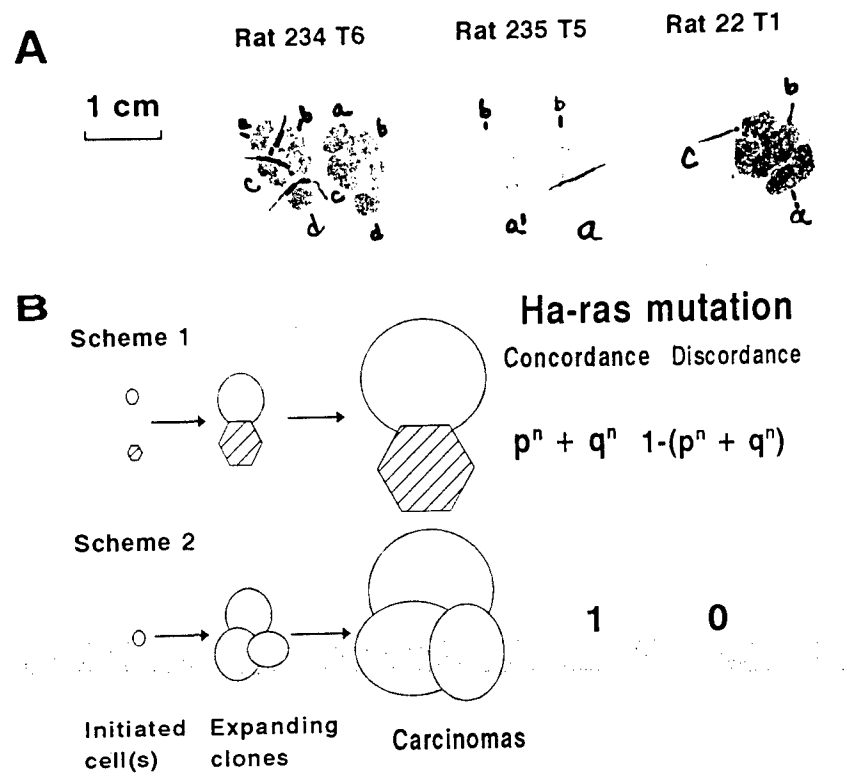
A
Rat 234 T6
Rat 235 T5
Rat 22 T1
1 cm
B
Scheme 1
Ha-ras mutation
Concordance
Discordance
$p^n + q^n$
$1-(p^n + q^n)$
Scheme 2
1
0
Initiated cell(s)
Expanding clones
Carcinomas
C
Rat 234
Rat 235
Rat 22
P N T1 T2 T3 T4 T5 T6 a b c d T7 T1 T2 T3 T4 T5 a b T1 a b c

Figure 1

MANUSCRIPT 3

Retention of Conjugated Linoleic Acid in the Mammary Gland is Associated With Tumor Inhibition During the Post-Initiation Phase of Carcinogenesis

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Abstract

Conjugated linoleic acid (CLA) has been reported to have significant activity in inhibiting mammary carcinogenesis. A major objective of this study was to evaluate how the kinetics of CLA retention in mammary tissue as a function of CLA exposure/withdrawal was correlated with cancer prevention in this target organ. Rats treated with a single dose of dimethylbenz(a)anthracene (DMBA) at 50 days of age were given 1% CLA in the diet for either 4 weeks, 8 weeks or continuously following carcinogen administration. No cancer protection was evident in the 4 or 8 week-CLA treatment groups. Significant tumor inhibition was observed only in rats that were given CLA for the entire duration of the experiment. Analysis of CLA in the mammary gland showed that the incorporation of CLA was much higher in neutral lipids than in phospholipids. When CLA was removed from the diet, neutral lipid- and phospholipid-CLA returned to basal values in about 4 and 8 weeks, respectively. The rate of decay of neutral lipid-CLA (rather than phospholipid-CLA) subsequent to CLA withdrawal paralleled more closely the rate of emergence of new tumors in the target tissue. It appears that neutral lipid-CLA may be a more sensitive marker of tumor protection than phospholipid-CLA. However, the physiological relevance of CLA accumulation in mammary lipids is unclear and remains to be determined. A secondary goal of this study was to investigate whether CLA might selectively inhibit clonal expansion of DMBA initiated mammary epithelial cells with wild type versus codon 61 mutated *Ha-ras* genes. Approximately 16% of carcinomas in the control group (without CLA) were found to express codon 61 *ras* mutation. Although continuous treatment with CLA reduced the total number of carcinomas by 70%, it did not alter the proportion of *ras* mutant versus wild type

carcinomas. Thus based on analysis of the *ras* genotype marker, the data seem to suggest that a clonal selection process might not be involved in tumor suppression by CLA.

Introduction

Conjugated linoleic acid (CLA) is a minor fatty acid found preferentially in red meat and dairy products (1). The biosynthesis of CLA in ruminants is accounted for by a rumen bacterium which is known to convert linoleic acid to stearic acid via CLA (2). Over the past decade, research from several laboratories has shown that CLA expresses powerful activity in cancer protection in a number of animal models (3-7). Feeding diets containing ≤ 1% CLA results in a dose-dependent suppression of tumor development in the mammary gland (8). CLA appears to have a dual effect in the modulation of mammary carcinogenesis in rats. First, exposure to CLA during the window of active mammary gland morphogenesis may reduce the proliferation of epithelial end bud cells, thus conceivably rendering the target cell population less susceptible to carcinogen-induced neoplastic transformation (8,9). Second, CLA is also capable of inhibiting tumor promotion/progression (9); however, a continuous supply of CLA is required for this mechanism of action.

The above study regarding the effectiveness of CLA in blocking tumor progression was carried out in the methylnitrosourea (MNU)-induced mammary carcinogenesis model in rats fed a 5% corn oil diet (9). One objective of the experiments reported in this study was to confirm the necessity of maintaining CLA intake after cancer induction by using dimethylbenz(a)anthracene (DMBA)-treated rats fed a 20% corn oil diet. It was considered important to assess whether the requirement for continuous CLA feeding was dependent on the nature of the carcinogen or the fat content of the diet. Rats were therefore given CLA for a duration of either 4, 8 or 20 weeks starting immediately after a single dose of DMBA to evaluate the anticarcinogenic efficacy of these various intervention regimens. The kinetics of mammary tissue CLA retention as a function

of CLA exposure/withdrawal was also analyzed in order to determine the correlation between time-dependent changes in tissue concentrations of CLA and effectiveness of cancer protection. Additionally, we were interested in finding out whether CLA might selectively inhibit the clonal expansion of DMBA-initiated cells carrying either the wild type or codon 61 mutated *Ha-ras* gene. Previous work from Thompsons' laboratory has shown that high dietary levels of linoleic acid preferentially increased the number of wild type *Ha-ras* mammary tumors, but not the codon 12 mutant *Ha-ras* tumors, in the rat MNU model (10). In chemical carcinogenesis, specific *ras* mutations are induced and are believed to be involved in early stages of tumor development (11-14). Generally *ras* mutation is considered to be permissive but not sufficient for carcinogenesis. Thus the *ras* genotype was used as a marker in the present study to identify subpopulations of neoplastically transformed cells that might be differentially modulated by CLA intervention.

Materials and Methods

Pathogen-free female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories at 45 days of age. They were fed a 20% corn oil diet (6) and were intubated with a single dose of 10 mg of DMBA at 50 days of age for the induction of mammary tumors. Supplementation of CLA (Nu-Chek, Elysian, MN) at 1% in the diet was started 4 days after carcinogen administration. A total of 90 rats were given CLA and were divided equally into 3 groups according to the length of CLA treatment: 4 weeks, 8 weeks or continuous until the end of the experiment. Control rats (n=30) were not given CLA at any time during the study.

Animals were palpated weekly for mammary tumors; the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 20 weeks after DMBA. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by chi-squared analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis as described previously (15).

A total of 177 mammary adenocarcinomas were harvested from the above carcinogenesis bioassay. They were individually identified after exercision so that each one could be tracked to its time of appearance in a particular rat. All 177 paraffin block-embedded tumors were analyzed for codon 61 *ras* mutation (CAA→CTA) by a modification of the polymerase chain reaction-generated restriction fragment length polymorphism (PCR/RFLP) method as described by Kumar and Barbacid (16). Two 5-micron sections were prepared side-by-side from the same paraffin block, one mounted on a plastic slide, the other on a glass slide which was subsequently stained

with hematoxylin and eosin for the identification of tumor cell foci under the microscope. The exact same area of interest was matched on the plastic slide and was then cut out for DNA extraction (17). The primers used for PCR amplication were 5'-GAGACGTGTTTACTGGACATCTT-3' and 5'-GTGTTGTTGATGGCAAATACACAGAGG-3' (synthesized by Integrated DNA Technologies, Coralville, IA), which yielded a 116 bp PCR product (18,19). The PCR reaction mixture contained 5 μl of DNA extract, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 15 μM deoxynucleotide triphosphate, 1 μCi of α-[^{32}P]dCTP, 0.1 μM upstream and downstream primers, and 0.5 units AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT). For each batch of PCR reaction, PCR-grade H_2O was used as a blank, DNA from a tumor bearing *Ha-ras* codon 61 mutation as a positive control, and DNA from normal mammary gland as a negative control. Amplification was carried out for 40 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min using a GeneAmp PCR system 9600 (Perkin Elmer). The codon 61 A→T mutation introduces a Xba I restriction site into the 116 bp PCR product, which upon digestion, generates two fragments of 80 and 36 bp that are diagnostic for the mutation. In contrast, the PCR product of the normal gene contains a sequence that is not susceptible to digestion by Xba I. The digested materials were separated by electrophoresis on a 6% polyacrylamide gel, and detected by autoradiography on X-ray film.

To study the kinetics of CLA retention in the mammary gland, a 2-part experiment was carried out to examine (a) the rate of increase of tissue CLA following the start of CLA feeding, and (b) the rate of disappearance of tissue CLA following CLA withdrawal. For the first part, 60-day old rats (age-matched to those in the above carcinogenesis experiment but not given DMBA) were fed a 1% CLA diet and were sacrificed at 1, 2, 4, 6 or 8 weeks later. For the second part,

rats were fed a 1% CLA diet for 8 weeks, the treatment was discontinued and necropsy was timed at 1, 2, 4 or 6 weeks after CLA withdrawal. Total lipid was extracted from the mammary gland (No. 4, 5 and 6) by chloroform/methanol. The separation of neutral lipids and phospholipids was achieved with the use of a Sep-Pak silica cartridge as described in an earlier publication (5). Gas chromatographic analysis of the CLA methyl ester was determined by the method reported previously by Chin et al (1).

Results

Figure 1 shows the time course of mammary tumor development in control rats or rats fed CLA for various lengths of time. It can be seen that short term feeding of CLA for only 4 or 8 weeks after DMBA administration was not effective in tumor inhibition. In the 8 week-CLA treatment group, the time course curve was shifted slightly to the right, suggesting a delay of about 2 to 3 weeks in the appearance of tumors. However, as soon as CLA was withdrawn, the rate of tumor appearance resumed at a rapid pace. At the time of necropsy, the difference in tumor occurrence between the control group and the 8 week-CLA treatment group was not statistically significant. In contrast, marked cancer protection, as judged by a 50% reduction in tumor incidence and a 70% reduction in the total number of tumors, was observed in rats that were given CLA for the entire duration of the study.

Figure 2 shows some representative electrophoresis autoradiograms of XbaI digested PCR products from tumors with either wild type or codon 61 mutant *ras* gene. The arrow in the diagram indicates the presence of a 80-bp band which is diagnostic for the mutation. Table I summarizes the appearance of each wild type or mutant *ras* mammary carcinoma along a timeline. In the control group, 16% of the tumors expressed the mutant *ras* gene. Continuous feeding of CLA reduced the total number of carcinomas by 70% but was found to suppress approximately the same proportion of wild type and mutant *ras* carcinomas in comparison to the control group. Short-term treatment with CLA for 4 or 8 weeks did not decrease significantly the total number of carcinomas, nor did it alter markedly the distribution of carcinomas carrying either the wild type or mutant *ras* gene. Overall, there was clearly no unusual pattern in the time of appearance of the *ras* mutant tumors due to CLA intervention. The slight delay in the emergence of this

tumor type as a result of 8 week- or continuous-CLA feeding was consistent with the delay in general of all the tumors in these two groups (see Fig. 1). Thus our results indicate that CLA inhibited carcinogenesis irrespective of the presence or absence of the codon 61 *ras* mutation.

Figure 3 shows the kinetics of CLA accumulation and disappearance in the neutral lipid fraction of the mammary tissue following CLA administration and withdrawal. As the results indicate, the incorporation was rapid once the animals were introduced to CLA in the diet. The level reached approximately 70% of maximum after 2 weeks of feeding, and plateaued after 4 weeks. At the peak, CLA was present at roughly 3% of total fatty acids in the neutral lipid fraction. In this experiment, some animals were given CLA for 8 weeks. The regimen was stopped, and the decay curve of CLA was then plotted in the same composite diagram. Figure 3 shows that as soon as CLA was discontinued, the rate of disappearance from the mammary tissue was equally fast, with a return to basal value in about 4 weeks time.

Figure 4 shows the increases and decreases of mammary gland phospholipid CLA from the same experiment. It should be noted that during CLA supplementation, the concentration of CLA in phospholipids (expressed as percent of total fatty acids) was, on the average, an order of magnitude lower than the concentration in neutral lipids. Interestingly, the rate of change of phospholipid CLA in either the upswing or downswing of the exposure/withdrawal curve was slower compared to that observed with neutral lipid CLA. After the start of CLA feeding, the maximum level in phospholipids was not attained until about 6 to 8 weeks later. Similarly, a diminished but still detectable amount of CLA was present by 6 weeks subsequent to the removal of CLA from the diet.

Discussion

The present study confirms our previous report that a continuous supply of CLA is necessary for maximum tumor inhibition in the post-initiation phase of mammary carcinogenesis. As pointed out in the Introduction, the first experiment was done in MNU-treated rats fed a 5% corn oil diet (9), whereas the repeat experiment described here was carried out in DMBA-treated rats fed a 20% corn oil diet. Thus this characteristic of CLA in chemoprevention is apparently not dependent on specific genomic mutation induced at the time of initiation or the availability of linoleic acid fed to the animals during tumor progression. It might be instructive to contrast the effects of CLA and linoleic acid at this point. Our study here indicated that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of *ras* mutation. Linoleic acid, on the other hand, has been demonstrated to promote selectively the development of the wild type *ras* tumors, but not the mutant *ras* tumors, in MNU-treated rats (10). Recent data also suggested that the response to CLA is unlikely due to a displacement of linoleic acid in the mammary tissue (20). Collectively, the above information provides supportive evidence that these two fatty acids may have distinctive mechanisms in the modulation of mammary carcinogenesis.

Mutations of the *ras* gene have been reported to occur in a target organ- and chemical carcinogen-specific manner in a number of experimental models (21). Zarbl et al (22) have previously described that in MNU-induced mammary tumors, GGA→GAA mutation in codon 12 of the *Ha-ras* protooncogene is a common event. The mutation probably results from methylation of guanine by diazomethane, a spontaneous decomposition product of MNU. In contrast, these same investigators found that only 21% (3 out of 14) of DMBA-induced mammary tumors express a CAA→CTA mutation in codon 61 of the *Ha-ras* gene (22). It has been

proposed that the A→T transversion is likely due to affinity of the DMBA diol epoxide to adenine residue as well as to sequence selectivity in binding of the metabolite to the *Ha-ras* DNA (23,24). To our knowledge, there has been one other study examining *Ha-ras* codon 61 mutation in the DMBA model. Interestingly, Waldmann et al (25) did not find such a mutation in a total of 50 tumors. The relatively low incidence of *Ha-ras* codon 61 mutation in our study is similar to that reported by Zarbl et al (22). However, it should be noted that our analysis was done in a much larger sample size. In any case, the data in Table I clearly indicate that DMBA-initiated cells with or without a *Ha-ras* codon 61 mutation are equally sensitive to the inhibitory activity of CLA.

Recent studies by Banni et al (26) have shown that in rats fed only 0.04% CLA in the diet for 1 week, conjugated diene-C18:3 and -C20:3 were recovered in the liver. Thus it appears that desaturation and elongation of CLA can occur in vivo while maintaining the conjugated diene structure. The presence of a conjugated diene-C20:4 metabolite could compete with arachidonic acid for the cyclooxygenase and lipoxygenase enzymes, thereby altering the biosynthesis of prostaglandins, thromboxanes and leucotrienes. These downstream products of arachadonic acid have been implicated by many investigators to be associated with promotion of carcinogenesis (27-32). By acting as a precursor to conjugated diene-C20:4, CLA could potentially play the role of a metabolic modulator in this process. Thus it becomes imperative to determine if conjugated diene-C20:4 is found in the mammary gland and if it is compartmentalized in a specific lipid fraction.

From our CLA analytical data, it is tempting to postulate that neutral lipid CLA may be a better indicator of protection than phospholipid CLA. Neutral lipid is far more plentiful than phospholipid in the mammary gland (see our previous work quoted in reference 6). The larger

pool of CLA in the former fraction may be more responsive to dietary intake because it serves as a depot for fatty acids that are not immediately utilized. Furthermore, the rate of decay of neutral lipid CLA following CLA withdrawal (Fig. 3) seems to match more closely the rate of emergence of new tumors (refer to 4 week- or 8 week-CLA groups in Fig. 1).

At first glance, the data on the kinetics of CLA retention in the mammary tissue appear to provide a reasonable explanation of why an uninterrupted supply of CLA is necessary to achieve tumor inhibition. As long as there is an abundant source of CLA present in the target organ, tumor appearance will be blocked or delayed. However, one must not lose sight of the possibility that CLA or a metabolite may induce an effect which is independent of its accumulation in mammary lipids. Future research will be aimed at delineating (a) whether neutral lipid and phospholipid CLA levels simply represent indicators of CLA exposure, (b) whether they serve as a local supply of CLA for further metabolism, and (c) whether different cellular compartments of the mammary gland are involved in the accumulation and metabolism of CLA that ultimately leads to cancer prevention.

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Table I. Time course of wild type and mutant *ras* mammary tumor appearance in control and CLA-supplemented rats[a]

Treatment	ras genotype	weeks after DMBA 5	6	7	8	9	10	11	12	13	14	15	16	17	total (%)	Median time of appearance (wk)
control	wild type	2	4	3	8	5	7	5	6	3	2	1	2	1	49 (84%)	9.2
	mutant	0	2	1	2	0	2	1	1	0	0	0	0	0	9 (16%)	8.7
4 wk-CLA	wild type	0	1	3	7	9	11	7	3	3	1	3	1	0	49 (92%)	9.8
	mutant	0	1	0	1	0	1	0	1	0	0	0	0	0	4 (8%)	9.0
8 wk-CLA	wild type	0	1	1	0	2	9	8	12	4	3	1	2	0	43 (86%)	11.3
	mutant	0	0	0	0	0	1	3	1	0	2	0	0	0	7 (14%)	11.3
continuous-CLA	wild type	0	0	1	3	2	1	1	2	1	0	1	1	0	13 (81%)	10.5
	mutant	0	0	0	0	0	2	1	0	0	0	0	0	0	3 (19%)	10.5

[a] These tumors were harvested from the mammary carcinogenesis experiment described in Fig. 1.

Figure Legend

Fig. 1. Effect of interrupted versus continuous CLA feeding after DMBA administration on mammary carcinogenesis. The duration of CLA feeding in the 3 supplemented groups is indicated along the X-axis time line by the filled symbols, which match the time course of mammary tumor development on the main body of the diagram. Control group without CLA supplementation is represented by the open circle. The asterisk denotes statistically significant difference ($P<0.05$) from the control data.

Fig. 2. Detection of *Ha-ras* codon 61 CAA→CTA mutation by PCR/RFLP method. The mutation produces a XbaI site in amplified 116-bp DNA fragment. Upon separation of the XbaI-digested product in 6% polyacrylamide gel, the presence of a 80-bp band (arrow) serves as a diagnostic marker for the mutation. PCR products were labeled with tracer amount of α-[^{32}P]dCTP and detected by autoradiography. Lane B, blank without template DNA; Lane M, normal mammary gland DNA as a negative control; Lane 1-16, mammary adenocarcinomas DNA. + and –, 5 μl of PCR product treated with or without 5 units of XbaI, respectively.

Fig. 3. The kinetics of CLA retention in neutral lipids of mammary gland following CLA supplementation and withdrawal. The results are expressed as % of total fatty acids, mean ± SE (n=6).

Fig. 4. The kinetics of CLA retention in phospholipids of mammary gland following CLA supplementation and withdrawal.

Figure 1

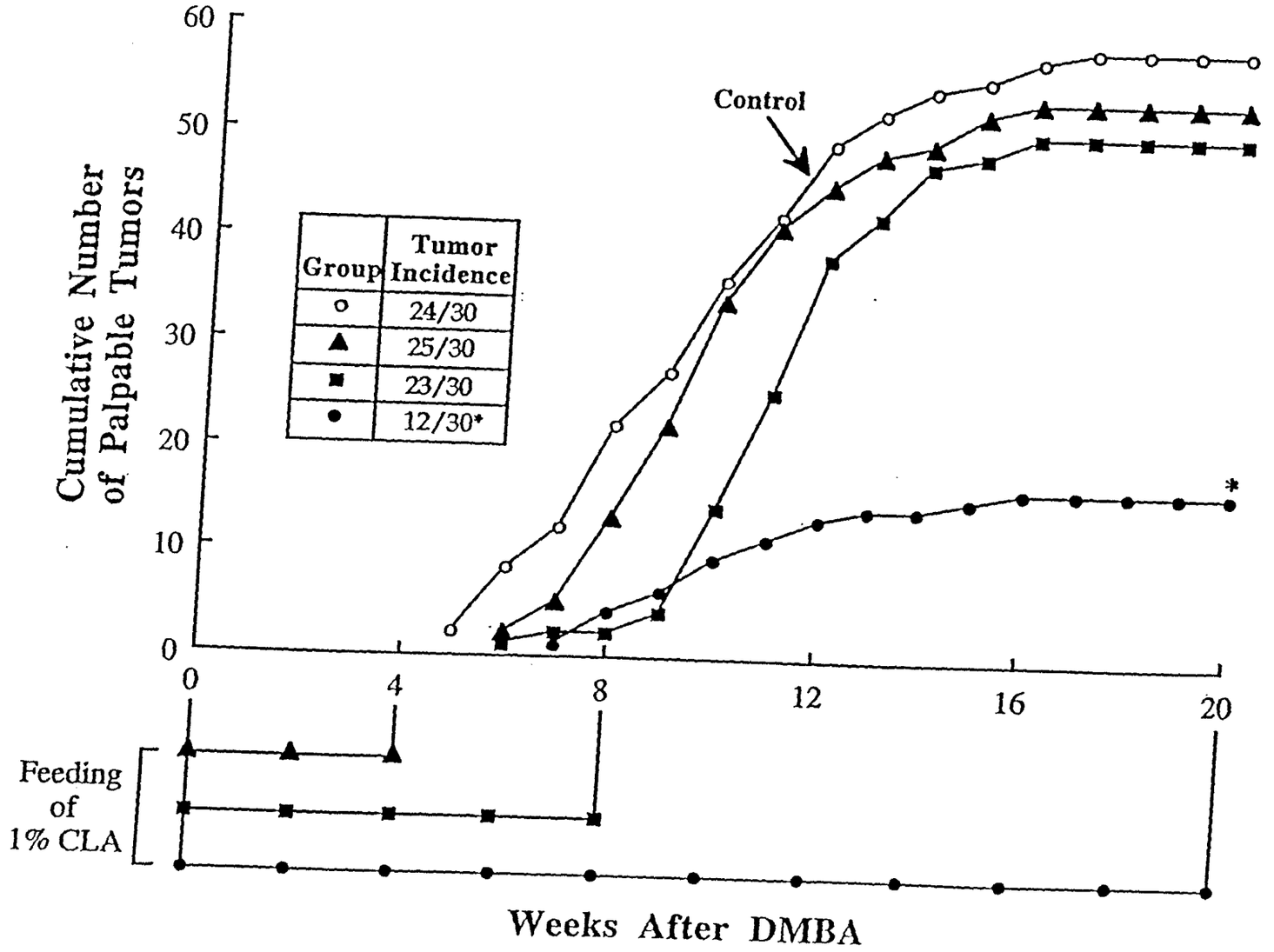

Cumulative Number of Palpable Tumors
60
50
40
30
20
10
0
Control
Group
Tumor Incidence
24/30
25/30
23/30
12/30*
*
0
4
8
12
16
20
Feeding of 1% CLA
Weeks After DMBA

Figure 2

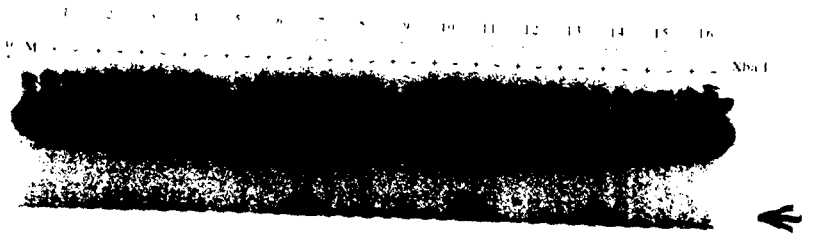
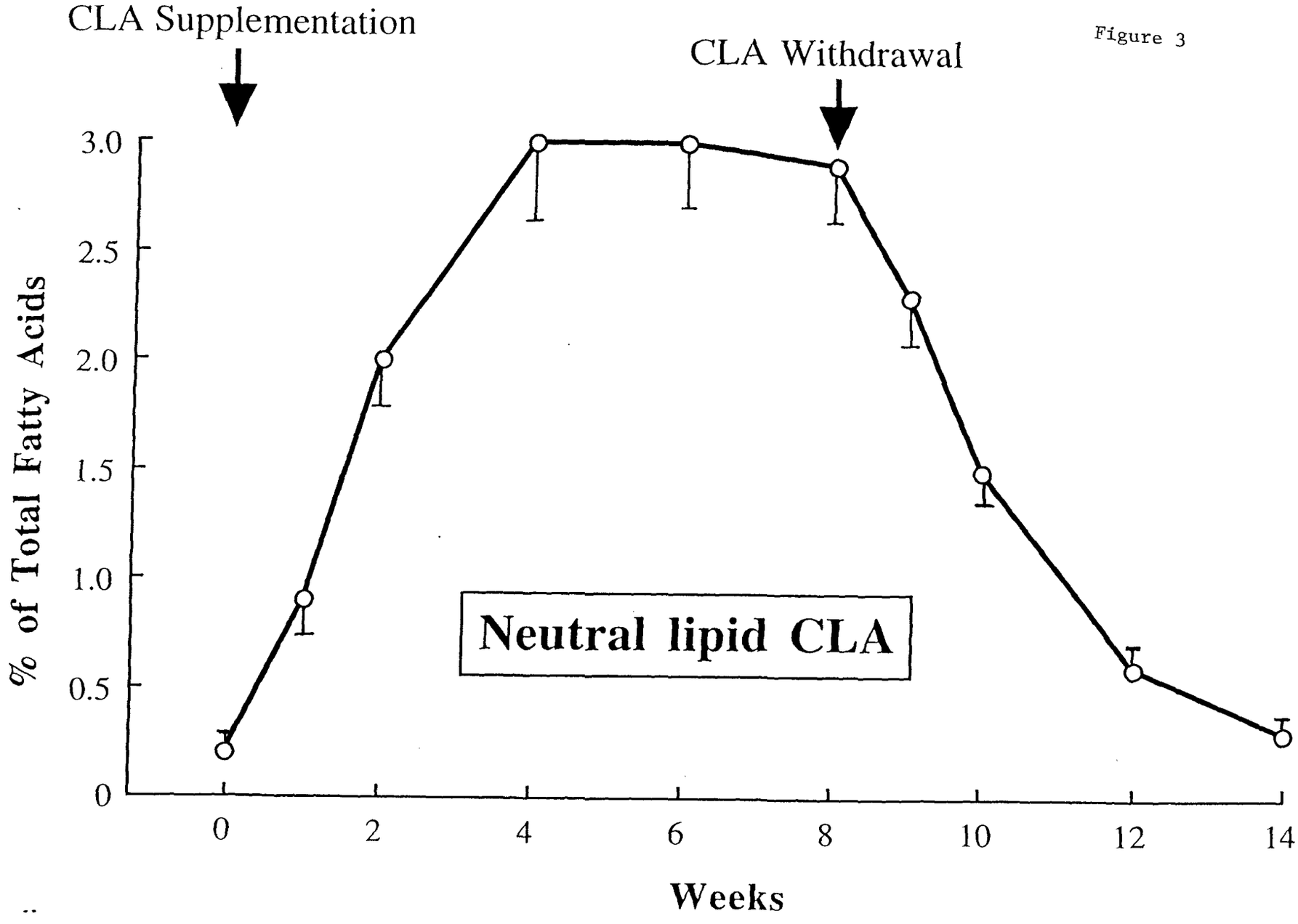


Figure 3


CLA Supplementation
CLA Withdrawal
3.0
2.5
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1.5
1.0
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% of Total Fatty Acids
Neutral lipid CLA
0
2
4
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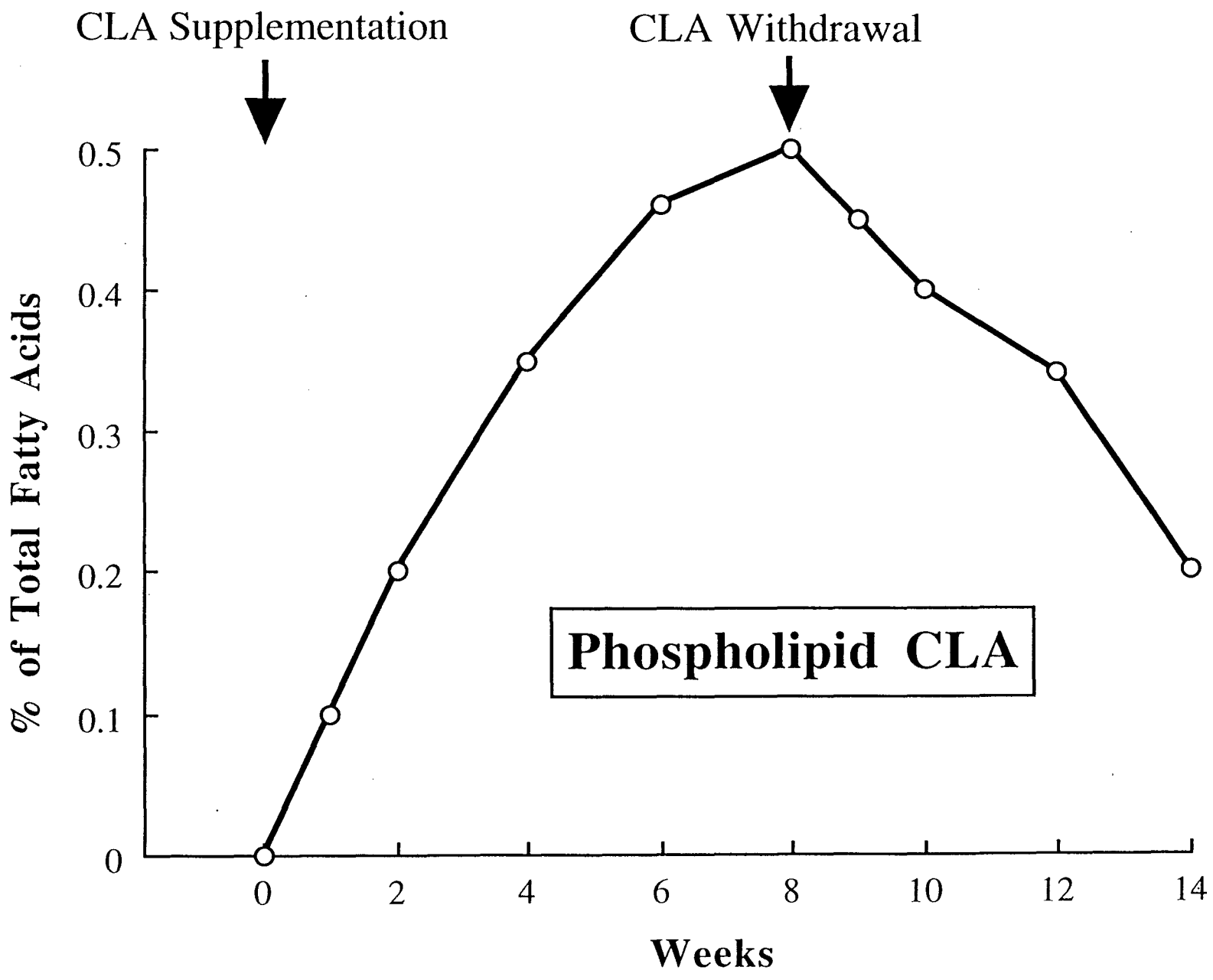
14
Weeks

Figure 4


CLA Supplementation
CLA Withdrawal
0.5
0.4
0.3
0.2
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% of Total Fatty Acids
Phospholipid CLA
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2
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6
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14
Weeks

MANUSCRIPT 4

GENE EXPRESSION CHANGES ASSOCIATED WITH CHEMICALLY-INDUCED RAT MAMMARY CARCINOGENESIS[1]

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Running title: Gene expression markers of rat mammary carcinogenesis

Key words: mammary chemical carcinogenesis, gene expression, differential display, cancer markers, molecular cloning

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ABSTRACT

Chemically-induced models of mammary carcinogenesis in the rat are widely used to study the biology of breast cancer development and to examine potential approaches for the prevention of this human disease. Whereas both genetic mutations and alterations in gene expression are being elucidated in the human disease, very little is known about gene expression changes that are associated with chemically-induced carcinogenesis in animal models. This paper reports the identification of over-expressed genes associated with mammary carcinogenesis induced by 1-methyl-1-nitrosourea using differential display of mRNA and molecular cloning. Nine over-expressed gene fragments were identified in mammary carcinomas in comparison with the uninvolved mammary gland, liver and kidney. Clone 3 represented the rat homologue of human galectin-7. Clone 4 was the homologue of the gene coding for the human melanoma inhibitory activity protein/bovine chondrocyte-derived retinoic acid sensitive protein (MIA/CD-RAP). Clone 16 and 18 were the homologues of the mouse/human DNA primase small unit and mouse stearyl-CoA desaturase-2 gene, respectively. Clones 10 and 14 were the homologue of mouse endo B cytokeratin/human cytokeratin-18 gene. Although these known genes have each been implicated in some aspect of carcinogenesis, their identification in this study is the first time that they have been associated with chemically induced mammary carcinogenesis. Clone 15 did not match any gene or expressed sequence tag (EST) whereas clones 1 and 9 showed significant homology to several ESTs. The expression patterns of clones 3,

4,10/14 and 18 were highly mammary carcinoma-specific when compared to 12 other normal tissues such as either virgin or lactating mammary gland, liver, heart and lung. The tumor-specific expression of these genes was also observed in rat mammary carcinomas induced by 7,12-dimethylbenz(alpha)anthracene (DMBA), indicating the generality of the role of the identified genes in rat mammary carcinogenesis. Full length cDNA has been cloned and sequenced for clone 3 and clone 4. Sequence comparison between cDNA obtained from rat fetal tissue RNA and that from mammary carcinoma RNA revealed no mutation in the MIA/CD-RAP coding region in the MNU-induced mammary carcinomas. The gene expression changes identified in this study may represent useful molecular markers to understand mammary carcinogenesis in these model systems as well as in the human disease.

INTRODUCTION

Breast carcinogenesis, as cancer development in other organ sites, is a multistep process that spans decades. Qualitative genetic changes such as gene mutations and chromosomal alterations as well as quantitative gene expression changes have now been found to be quite common in malignancies. Although the recent discovery of breast cancer susceptibility genes BRCA-1 and BRCA-2 (1-3) highlights the significant progress toward understanding the basis of genetic susceptibility to breast carcinogenesis, the elucidation of the patho-genetic pathways and the identification of molecular markers of sporadic breast carcinogenesis, the majority of which do not involve these two susceptibility genes, still remain an unattained goal.

As a step toward that goal, we have undertaken the identification of gene expression changes associated with a widely used model of breast carcinogenesis, i.e., the 1-methyl-1-nitrosourea (MNU)-induced mammary carcinogenesis in the rat by differential display of mRNA (4) and molecular cloning. The underlying hypothesis was that quantitative changes in the expression of critical genes contributed to the genesis of mammary cancer and such genes were potential molecular markers of mammary carcinogenesis. Here we report the identification of 9 over-expressed gene fragments associated with mammary carcinogenesis induced by MNU and the expression patterns of selected genes in normal tissues as well as in mammary carcinomas induced by either MNU or 7,12-dimethylbenz(alpha)anthracene (DMBA) in the rats.

MATERIALS AND METHODS

Chemical Carcinogenesis MNU-induced rat mammary carcinomas that were used for the differential display of mRNA were obtained by a recently published short-term protocol of Thompson et al. (5). Briefly, female Sprague-Dawley rats were obtained from Taconic Farms, Germantown, NY at 21 days of age and were given an i.p. injection of MNU (50 mg per kg body weight). At two months post-MNU injection, a rat (#35) bearing multiple mammary tumors was killed and 3 carcinomas, the uninvolved mammary glands and the kidney were quickly excised and frozen in liquid nitrogen immediately. In order to establish the relevance of the genes identified from this short term model to the "conventional" MNU carcinogenesis model in which the carcinogen is administered at 50 days of age, mammary carcinomas were also obtained by an i.p. injection of MNU at 50 days of age (6) and were used for gene expression detection by Northern blot analyses. In addition, DMBA-induced rat mammary carcinomas were obtained from Dr. Clement Ip of Roswell Park Cancer Institute, Buffalo, NY to examine the generality of these gene expression changes in rat mammary carcinomas induced by two different chemical carcinogens.

RNA isolation Total RNA was extracted from carcinomas and tissues by acidic phenol extraction using a commercial kit from BIOTECX Laboraotries, Inc. (Houston, TX). For differential display of mRNA, the total RNA preparations were digested with RNase-free DNase (GenHunter Corporation, Nashville, TN) in order to remove contaminating genomic DNA. For cDNA library construction, poly A(+)

mRNA was enriched by oligo (dT)-cellulose column.

Differential display Differential display of mRNA was carried out with the RNAimage™ kit (GenHunter Corporation, Nashville TN) according to manufacturer's instructions with two minor modifications: 1) One tenth of the recommended amount of total RNA was used for the reverse transcription step in order to minimize an inhibitory activity(s) present in the mammary tissue RNA preparation; 2) It was found that an annealing temperature of 42 °C for PCR was optimal in Denver, CO to yield reproducible display patterns. Duplicate reactions were run for each primer combination. The PCR products (labeled by alpha-^{32}P-dATP) from the 3 mammary carcinomas (T_1, T_2 and T_3), the uninvolved mammary tissue and kidney were contrasted side-by-side on sequencing gels. Only those bands that were present in carcinoma lanes, but absent in mammary gland and kidney lanes were cut and re-amplified by PCR. The PCR products were size separated on low melting point agarose gel and band(s) of the expected size was eluted. The gel-purified PCR bands were used as templates to generate ^{32}P-labeled probes for Northern blot detection of gene expression on a screening panel of RNA preparations comprised of two kidney samples, two liver samples, the 3 mammary tumors that were used for the original differential display. In addition, the uninvolved mammary gland tissue and mammary gland tissue excised from a day-1 post-partum female rat were included in the screening panel. The RNA samples were size-separated by electrophoresis and transferred onto Nylon membrane for Northern blot detection of gene expression. GAPDH or cyclophylin gene was

probed as an internal control for loading correction.

Cloning and Sequencing Those PCR bands that detected carcinoma-specific gene expression were cloned into pGEM-T vector (Promega, Madison, WI). For each band, four clones were inoculated and the plasmid DNA was isolated by an alkaline mini-prep procedure and at least two clones are sequenced on both strands by the dideoxy chain termination method of Sanger (7) using a kit from US Biochemicals (St Louis, MO). A commercial service utilizing thermal cycle sequencing (Cornell DNA Service, Ithaca, NY) was also used to confirm the sequence of a few of the clones. The cloned gene fragments were used as templates to generate randomly labeled probes for Northern detection again to confirm that the cloned sequences corresponded to the genes originally detected by the PCR products from differential display gels. Sequence search was done using the BLASTN algorithm (8) with GenBank nr databases and expressed sequence databases dbEST.

Cloning full length cDNA A cDNA library was constructed with pooled poly(A)+ mRNA isolated from mammary carcinomas using the Marathon cDNA construction kit (Clontech, Inc, Palo Alto, CA). The average length of the library was 1.5 kb. Based on the sequence information obtained for each gene fragment, a gene-specific primer was synthesized (Integrated DNA Technology, Inc, Coralville, IA) as the down stream primer. A universal upstream primer that annealed to the adapter which had been ligated into the cDNA library and the gene-specific primer were used for long distance PCR using KlenTaq (a combination of Taq and Vent polymerases) to increase fidelity of cloning (ClonTech, Palo Alto, CA). The PCR fragments were cloned into the pGEM-T vector and sequenced as described above.

RESULTS

1. Differentially expressed gene fragments

A total of 15 primer pair combinations were used to compare the gene expression patterns of 3 MNU-induced mammary carcinomas with those of the uninvolved mammary gland tissue and the kidney, all of the same rat. Of 21 bands analyzed, 16 represented differentially expressed genes between the mammary carcinomas and normal tissues. This high level of positivity (i.e., 76% = 16/21) for identifying gene expression changes was attributed to the inclusion of a kidney sample in the differential display gels as an epithelium-rich control. This helped to rule out a great number of epithelium-related genes which could show differences between the mammary carcinomas and the mammary gland because of the large difference in epithelial cell proportion between these two sites.

Nine clones have been characterized in more detail and are reported here. Representative Northern blot detection of expression of these genes is shown in Figure 1. Several points are noteworthy. 1) Most of the clones detected tumor-specific expression with the exception of Clones 9 and 16, which detected some expression (<1/10 of the level in mammary carcinomas) in liver and virgin mammary gland, respectively. 2) The observed tumor-specific gene expression was not due to a difference in the proportion of mammary epithelial cells between the mammary carcinomas and the virgin mammary gland, which is comprised of ~ 5% epithelial cells. This was supported by the lack of expression in the mammary gland tissue that was obtained from a day-1 post-partum rat, even though the gland was comprised primarily of secretory epithelium at this stage of gland development

(Lane 9, Figure 1). 3) Some clones showed variable expression levels among mammary tumors (e.g., Clone 1 and Clone 18) from the same animal.

The sequences of the cloned gene fragments that detected differential changes by Northern blot analyses are shown in Figure 2. Sequence homology search by Blastn on GenBank nr databases identified homologues of several known genes (Table 1). These included clone 3, human galectin-7 (9); clone 4, human/mouse melanoma inhibitory activity (10)/bovine chorocyte-derived retinoic acid sensitive protein (11); Clones 10 and 14, which differed by 5 bases, mouse endo B cytokeratin (12)/human cytokeratin-18 (13); clone 16, mouse/human DNA primase small subunit (14,15) and clone 18, mouse stearyl Co-A reductase 2 (16). Comparison with the Genbank expressed sequence tag database (dbEST) identified significant homology between Clone 1 and two mouse clones (gb AA014143; gb W36666) and a human EST clone (gb N25349). Clone 9 had significant homology to human EST gb N35187 and to several mouse EST clones (gb W80253; gb W82774). No homology was found for Clone 15 in the databases searched.

2. **Tissue-specific gene expression**

The expression patterns of Clones 3, 4, 10/14 and 18 in non-mammary tissues were examined by Northern blot (Figure 3). Clear-cut mammary carcinoma-specific expression was observed for Clones 4 and 10/14 when compared with 12 other non-tumor tissues. Besides strong expression levels in the mammary carcinomas, some expression was observed for Clone 3 in the stomach ($<1/10$ of expression level in carcinomas) and Clone 18 in the brain ($\sim 1/5$ of expression level in carcinoma).

3. Expression pattern in rat mammary carcinomas induced by the "conventional" MNU injection protocol and by DMBA.

Figure 4 shows the expression patterns of Clones 3, 4 and 10/14 in mammary carcinomas induced by the conventional MNU induction protocol (i.e., at 50 days of age) and by DMBA. These data provided strong evidence of expression of these genes in the rat carcinomas regardless of the induction protocol and the nature of the chemical carcinogens used. Because many known tumor-specific antigens represent de-repressed expression of embryonic genes, the level of expression of these genes was examined in RNA extracted from mid-term (12-days) rat fetuses (Lane F, Figure 4). The expression level was below the limit of detection for all three genes, in spite of the fact that full length rat MIA/CD-RAP cDNA (Clone 4) was successfully cloned by RT-PCR from this RNA extract (see below).

4. Full length cDNA

The full length sequences have been obtained for Clone 3 (Figure 5, GenBank Accession No. U67883) and Clone 4 (Figure 6, GenBank accession No. U67884). Full length Clone 3 was 76% homologous to human galectin-7 at the nucleotide level and shared 72% identity and 84% positivity (i.e., similar charge characteristics) at the amino acid level (Figure 5). With respect to clone 4, i.e., MIA/CD-RAP, cDNA was also cloned from both a mid-term and a full-term fetal rat RNA preparation by RT-PCR and the sequences compared with that derived from the mammary carcinomas. No mutation was detected in the coding region of the cDNA cloned from the carcinomas. The deduced rat MIA/CD-RAP protein sequence was aligned with those of the human and other species in Figure 6. In

spite of some variability in the signal peptide region among species (the first 22-24 amino acids), the mature MIA proteins are highly conserved (94% identity with both human and mouse MIA, 90% identity with bovine CD-RAP).

DISSCUSSION

Expression data have been presented on 9 gene fragments that were over-expressed in the mammary carcinomas induced by the chemical carcinogen MNU, supporting the hypothesis that carcinogen-induced alterations of gene expression contribute to the transformed carcinoma phenotype. Several points are noteworthy concerning these genes: 1) None of the known homologues of the genes identified in this study have previously been associated with mammary carcinogenesis; however, each of these known genes or gene families has been implicated in some aspect(s) of carcinogenesis in other tissues. For example, the human melanoma inhibitory activity (MIA) protein was purified from the conditioned medium of a slow growing melanoma cell line as a secreted factor. Molecular cloning confirmed that the gene product is a secretory protein with inhibitory activity against melanoma cells in culture (10). The expression of this gene appears restricted to melanoma (10, 30), but has recently been detected in cultured bovine condrocytes as coding for a retinoic acid sensitive protein (CD-RAP) and in fetal rodent skeletal cartilage tissues (11). Sequence comparison carried out in this study between MIA/CD-RAP cloned from mammary carcinomas and from rat fetuses did not reveal any mutation in the coding region, supporting a role of altered MIA/CD-RAP gene expression rather than specific gene mutation in

chemically-induced rat mammary carcinogenesis. The fact that retinoic acid is a morphogen in embryogenesis and fetal development suggests a role of this gene in mediating morphogenesis, tissue remodeling and differentiation as well as in mammary carcinogenesis.

The galectin family of proteins share a common property in binding to galactosyl moiety with conserved primary structural features (17,18). Whereas little is known about the specific function of galectin-7, the human form of which was cloned in 1995 (9), galectin-1 and galectin-3 have been examined as tumor-specific markers in several organ sites and appear to be informative (19-25). While each type of galectin may have specific biological and pathological functions, their galactosyl binding property suggests that they may be involved in mediating cell-cell recognition and cell-matrix interaction, processes that are being increasingly recognized as to imparting significant regulatory controls over cell fates and tissue size homeostasis.

The detection of over-expression of the DNA primase small subunit (clone 16) in mammary carcinomas may reflect the increased DNA replication and cell proliferation in the transformed cells. Similarly, over-expression of stearyl-CoA reductase-2 (clone 18) gene may be indicative of altered lipid metabolism in mammary carcinoma cells. Endo B keratin/cytokeratin-18 and its partner Endo A keratin/cytokeratin-8 are extremely early embryonic genes, detectable at 4- or 8-cell stage of embryonic development in the mouse (26,27). In adult tissue, low level of gene expression, if any, is restricted to simple epithelium (28). Enforced over-expression of both cytokeratin-8 and cytokeratin-18, which are partners for

assembly into intermediate filaments, confers multiple drug resistance to mouse fibroblast in culture (29). The utility of these genes as potential gene expression markers for breast carcinogenesis remains to be determined.

2) The fact that expression changes of the genes identified in this study were observed not only in MNU-induced rat mammary carcinomas but also in DMBA-induced carcinomas indicates a generality of induction of these genes in rat mammary carcinogenesis. Unpublished data showed that in cultured mouse mammary epithelial cells, cytokeratin-18 (clone10/14) appeared to be expressed and the expression level correlated with the neoplastic transformation stage of the cell lines used (Lu et al, 1996). Work is in progress to evaluate the relevance of these genes in human breast carcinogenesis.

3) The newly identified mammary carcinogenesis associated genes do not contain retroviral sequences whereas 1 of 2 over-expressed genes identified by Young et al (31) in the same model system by subtractive hybridization is a retrovirus-related sequence. As the observed gene expression changes most likely represent a small fraction of altered gene expression associated with mammary carcinogenesis, different gene screening approaches are likely to complement one another to yield a fuller spectrum of the gene expression changes that are associated with mammary carcinogenesis.

In summary, the combination of differential display of mRNA and molecular cloning identified 9 gene fragments whose over-expression were associated with chemically induced rat mammary carcinogenesis. The expression of most of these genes was mammary carcinoma-specific. The fact that these genes were also

observed to be over-expressed in DMBA-induced mammary carcinomas indicated their potential broad utility as gene expression markers of mammary carcinogenesis.

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Table 1. Summary of gene fragments cloned by differential display of mRNA from MNU-induced rat mammary carcinomas

Clone No.	Fragment size bp.[1]	GenBank Accession No	Homologues identified by BLASTN search	% homology[2]
Clone 1	526	U67990	Mouse EST AA014143 Mouse EST W36666 Human EST N25349	234/250 = 94% 131/173 = 76% 138/178 = 78%
Clone 3	200	U67883	Human Galectin-7	91/124 = 73%
Clone 4	170	U67884	Human melanoma inhibitory activity MIA Mouse MIA	61/105 = 58% 96/106 = 90%
Clone 9	523	U67991	Mouse EST W82774 Mouse EST W80253	260/318 = 82% 143/166 = 86%
Clone 10	171	U67992	Mouse Endo B cytokeratin Human cytokeratin-18	102/116 = 87% 64/82 = 78%
Clone 14	166	U67992	Same as Clone 10, except missing 5 bp proceeding poly(A).	
Clone 15	390	U67993	Novel	
Clone 16	409	U67994	Mouse DNA primase small subunit, P49 Human DNA primase small subunit, P48	141/153 = 92% 60/77 = 88%
Clone 18	314	U67995	Mouse stearyl-CoA Desaturase-2	194/239 = 81%

[1]Fragment size excluding primer sequences.

[2]Homology was calculated based on the sum of stretches of DNA sequences that have been matched by BLASTN with GenBANK nr or Expressed sequence tag (EST) data bases.

Figure Legend

Figure 1. Northern blot analyses of gene expression detected by cloned gene fragments on a screening panel of RNA isolated from rat kidneys (lanes 1 & 2), 3 MNU-induced mammary carcinomas (lanes 3-5), livers (lanes 6 &7), an uninvolved virgin mammary gland (lane 8) and a lactating mammary gland at day 1 post-partum (lane 9). Arrowheads point to full size transcripts for clones 15 and 16 which showed gene-specific partial degradation of RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was probed as an internal control for loading correction.

Figure 2. Nucleotide sequences of cloned gene fragments. Single underlined sequences represent primers used for differential display of mRNA. Termination codons for known genes are double underlined. Canonical polyadenylation signals are highlighted in **bold**.

Figure 3. Northern blot detection of expression of selected genes in rat tissues and MNU-induced mammary carcinomas. Tissues examined were: S - spleen, L - liver, K - kidney, O - ovary, B - brain, St - stomach, SI - small intestine, C - colon, Lu - lung, H - heart, Mu - muscle, Mg - uninvolved virgin mammary gland and T_4, T_5, T_6 - mammary carcinomas induced by MNU using the short-term protocol. GAPDH gene was probed as an internal control for loading correction.

Figure 4. Northern blot analyses of gene expression in rat mammary carcinomas induced by MNU or DMBA. The carcinogens were administered when the rats were 50 days of age. T_2 and T_3 were RNA from MNU-induced mammary carcinomas used for the initial differential display. F was RNA isolated from mid-term rat embryos. Cyclophylin gene was probed as an internal control for loading correction.

Figure 5 Sequence of full length cDNA of rat galectin-7 and alignment of deduced amino acid sequence with the human homologue (9). A vertical line indicates amino acid identity and a plus "+" indicates similar charge characteristics for the encoded amino acids.

Figure 6 Sequence of full length cDNA of rat MIA/CD-RAP and alignment of deduced amino acid sequence with homologues from mouse (10), cow (11) and human (10). The reported rat partial sequence (11) was also aligned here for comparison. Predicted cleavage site for the signal peptide is between amino acid residues 22 and 23.

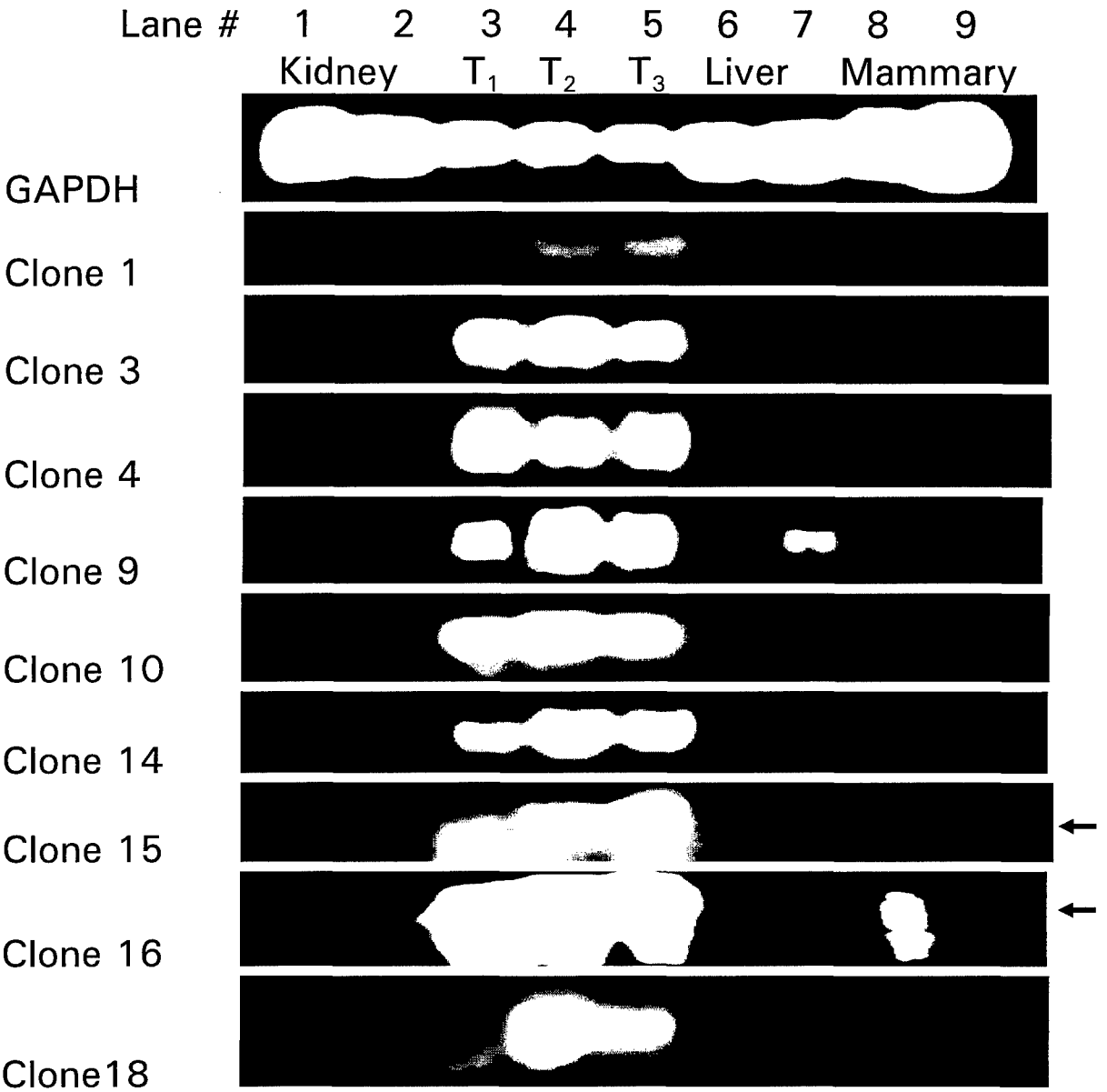
Lane # 1 2 3 4 5 6 7 8 9
Kidney
T1
T2
T3
Liver
Mammary
GAPDH
Clone 1
Clone 3
Clone 4
Clone 9
Clone 10
Clone 14
Clone 15
Clone 16
Clone18

Figure 1

CLONE 1 AAGCTTCGACTGT

1 CAAGGAACTA AAACCTTTAA AAGCATAGGC ATGCTGGCCT GAGGTAAACA CTGGTACAGT TAGAGCGGAG GCAGCAGCCC CGGTGTTCAG
91 CACAGCTTGT CTGATGTGTT TATGGCCAGA GTGCAGTATT CGGCACTGGC TAGTACCGCT GCCTGACCGA ACTCCACTGG GAAGGTTTTG
181 CTTAAACGCA CATGTTTCTT TGTTACTCCT GACCATGCTT GGATGCAGTG CCTANATTGT GGCTGCTATT TTTAGTTCGC ATCATGACCT
271 TGACTGCGGA GTCCAGCCTT ACCCTCTTAC TTGATAACAG TAGCCCTTAA ACTGCAGTGG GAAGAAAGAA GAATTTGGTA TGAAAATTGG
361 TGAGCTCTGG CACTTGAGAT AAACAAGAAG AAAGTTCCAA CTTGATGCCT TAAGCCGCTC CTGCCCGAAG CTCTGAAGGA ACTAAGTTGG
451 CAGAGATCCT TACTTTGATA CTACTGCATT TTTTGTAGAA TTGTTACATT GAT**AATAAA**A CTTGCCTGCT TAACCT CAAAAAAAAAAAGCTT

CLONE 3 AAGCTTCGACTGT

1 GATCGGGGAT GACGAGTATC TCCACTTCCA CCACCGGATG CCATCCTCTA ACGTGCGCTC AGTGGAGGTG GGCGGAGACG TGCAGCTGCA
91 TTCTGTGAAG ATCTTCTGAG CAAGGACCCA GGGGCTTGGC GAGTGGGGGT GGGGTTTCGT CAGATCGTAG AGGAGGGTTG TGGATGGCG**A**
181 **ATAAA** CTGTA GCTGTAGTTC CAAAAAAAAAAAGCTT

CLONE 4 AAGCTTCGACTGT

1 CAATGAGCTC AACCCACCGA TGTTATCCCT GCAGTTACCC TTCCGGTTTG GGCAAATACA GCGGCCAACT GCAAAGTGTT TTGTCCCTTT
91 GGTTTTTGGG GTGGGCATGT ACAAAGAATG TTTCACGGGT TCCTGAACCT AGCC**AATTAA** AGCCCTGAAT GTTGTAACGT
CAAAAAAAAAAAGCTT

CLONE 9 AAGCTTTGGTCAG

1 AGGTGCCAGA AGTGTTTTGG GTCTCAATTT GAGAGCCCTG AGTTCTCCAC AGAGAGCATT GAAAGGATGC TGAATAACCT GGTTAATTAT
91 ATTCTGCAAA GATACTACGG ACATAGGAAG ATAGCATCGA TCTTGAATGC ATCTTTGGAT GAAAAGGTGC TTTTGGGATG GGCCCCAAGA
181 ACAACATAAA ATGGGGAGGC ATGCAGTTCT TGCACCCCCA TGGAAGTGTG CCGTTGCACA GAGAGTGAGG CCACCCAGAT CCCCATCTCC
271 ATCTCCATCG CCATCACCAA AGAGCCACAG TTCCTCTCCA TCAAGGAGCC ACCCTCCTTT ACTTCAGACT G**R**GATTACAG GTTTTAGGAA
361 CAGGCCTTTT CAGGAAGTCA GAGAGCCCGG TGAAGCAGCA TCACCACTAT TAGTGATTTT GTCTGTCGCT GCGCTTGCCC TTATTGGTTT
451 CTTCATTAGA TAATAGGTTT ATCATCTTGT TTTAATCCCG TGGTGATC**AA TAAA**ATGGAT GTCATTTTAA CGT
GAAAAAAAAAAGCTT **R**=A OR G (polymorphic in two clones)

CLONE 10 AAGCTTCTCAACG

1 ACGCCCTGGA CTCCAGCAAC TCCTAGCAAA CTGCCAGAGG ACAACTACCC GTAAGGTCGT GGATGGCAAA GTGGTGTCCG AGACCAATGA
91 TACCAGAGTT CTGAGGCACT AAGGCTCAGA AGAAGGGAAC CCTTGGGGAC TGAGGGTCC**A ATAAA**AGTTT AGAATCCACT G
GAAAAAAAAAAAGCTT

CLONE 14 AAGCTTCTCAACG

1 ACGCCCTGGA CTCCAGCAAC TCCATGCAAA CTGCCAGAGG ACAACTACCC GTAAGGTCGT GGATGGNAAA GTGGTGTCCG AGACCAATGA
91 TACCAGAGTT CTGAGGCACT AAGGCTCAGA AGAAGGGAAC CCTTGGGGAC TGAGGGACC**A ATAAA**AGTTT AGAATC CAAAAAAAAAAAGCTT

Figure 2 (1 of 2)

CLONE 15 AAGCTTAGTAGGC

1 CAAGACACTT AGAGAAAATT ACTTTTTTGC ACTACTTCTT AAGCACAATT AGCAATCATC CATCACTTTC CAAAGAGTAG GTTTTCCAGT
91 TTGATTATCA TCTCAAATGC ATGAAATTTC TCTGATCAGT ATCACCACAG GAATCTGATC TCCATGTTCC TGGCTTTGTT TCAGTATAAA
271 TTGATGGAAT GGTATCACAG TCAATGGGGA GAAACGTTAC TTGTGACCAG CCATGTTCAA GTCAATTTAA CATTGGACTT TTTCCACAGC
361 TTCACCTCCA AAAAGGTGGA GGAAGGAGGT TTTCTGTTAG ATGGAAATAT GTGTTCTGCT ATGAATGTTA GTAACTGAGT AACTTTTGAA
451 ATATTTC**AAT AAA**AGTTGCC TTTTACACTA CAAAAAAAAAAAGCTT

CLONE 16 AAGCTTGCACCAT

1 ACGTGAAAGT ATTTGAACAG TTTCTCGAAA ACCTGGATAA ATCTCGGAAA GGGGCACTTC TCAAGAAGAG CGATTTACAA AAAGATTTCT
181 GAAGAAAACA ACCCCATCAA ACCAATGTGA ATGCCTTCTA CATTCAACCA TGGAGCATTT AGTCAATGCA GTAGAATCGT CAGTGTGACT
271 GAATCCTTAA AGCAGAGCTT TCTTTTCTTT TCTTTCTTTT TTCTTTTTTT CGGAGCTGGG GACGAACCCA GGGCCTTGAG CTTGTTAGGC
361 AAGCGCTCTA CCACTGAGCT GAATCCCCAA CCCCTCAGAG CTTTCTTTTG AGAAATCATT TATGTCAAAG AGACCTGGTT TCTGAGACCA
451 AAGAGGGTGT CAAAGGCCCT GAAAGAAAC**A ATAAA**TCAAG G**AATAAA**TG CAAAAAAAAAAAGCTT

CLONE 18 AAGCTTTGGTCAG

1 GGTAGGAGAT CTGAGATGCC GTGGACTTTG CAGAA**R**AGAT TTTGTGCAGC AACGCAGAAG CCTGGCTGTG TTAAGATTGG CTGTACAGGA
91 TAGCGAACTG TGGTTGGAGG GCACAGTCTT CCCGTGTTAG TTAAATAGAG AGGCTTTAGG ACGTCTCTGC GCCCACTTGA AGGATGCCTT
181 ATCCTTTAGC TGTTCGCTAA AATAGAATCC TTGAGAGATG ACAATATATT TGCTGCCTGT CGCCCTATTC CTTGGAGAGG CCTTTTGAAT
361 GGTTTTAATT CCACTGGTTA TCCGGTGCCC ACTTAATCAA ATGT GAAAAAAAAAAAGCTT **R**=A OR G (polymorphic in two clones)

Figure 2 (2 of 2)

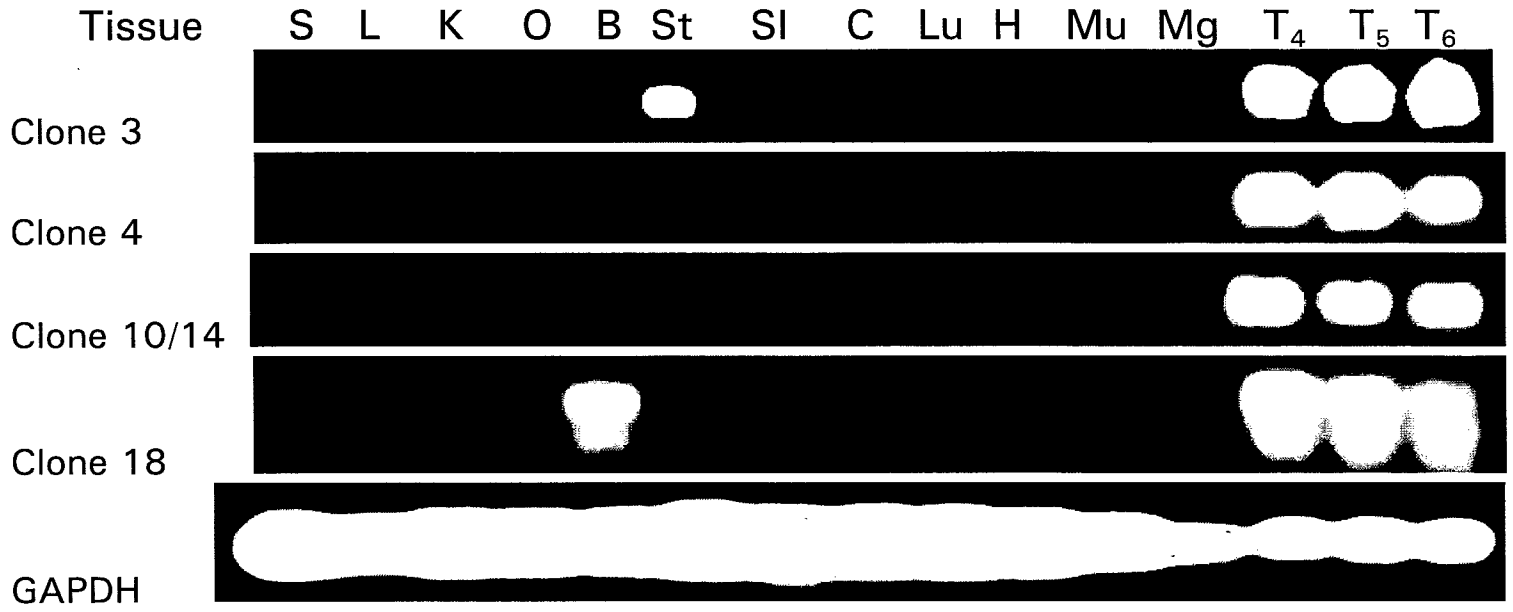
Tissue
S L K O B St Sl C Lu H Mu Mg T_4 T_5 T_6
Clone 3
Clone 4
Clone 10/14
Clone 18
GAPDH

Figure 3

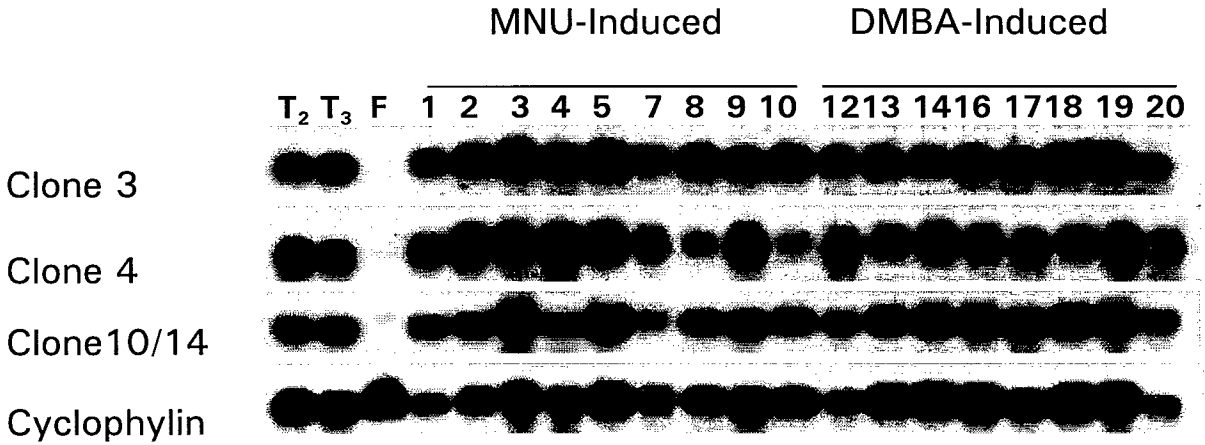
MNU-Induced
DMBA-Induced
T2 T3 F 1 2 3 4 5 7 8 9 10 12 13 14 16 17 18 19 20
Clone 3
Clone 4
Clone10/14
Cyclophylin

Figure 4

CLONE 3 **RAT GALECTIN-7**

5'-TTGC CGTGCCAGCC

```
15    ATG TCT GCC ACC CAT CAC AAG ACC CCT CTG CCT CAG GGT GTC CGC
1     Met Ser Ala Thr His His Lys Thr Pro Leu Pro Gln Gly Val Arg

60    CTG GGC ACC GTC ATG AGA ATT CGA GGC GTG GTC CCT GAC CAG GCT
16    Leu Gly Thr Val Met Arg Ile Arg Gly Val Val Pro Asp Gln Ala

105   GGC AGG TTC CAT GTA AAC CTG CTA TGC GGC GAG GAG CAA GAG GCA
31    Gly Arg Phe His Val Asn Leu Leu Cys Gly Glu Glu Gln Glu Ala

150   GAC TGC GCC CTG CAC TTT AAC CCG AGG CTG GAC ACA TCC GAG GTT
46    Asp Cys Ala Leu His Phe Asn Pro Arg Leu Asp Thr Ser Glu Val

195   GTC TTC AAC ACC AAA CAG CAA GGC AAA TGG GGC CGT GAG GAG CGG
61    Val Phe Asn Thr Lys Gln Gln Gly Lys Trp Gly Arg Glu Glu Arg

240   GGC ACC GGC ATC CCC TTC CAG CGT GGG CAG CCC TTT GAA GTG CTC
76    Gly Thr Gly Ile Pro Phe Gln Arg Arg Gln Pro Phe Glu Val Leu

285   ATC ATC ACC ACA GAG GAA GGC TTC AAG ACT GTG ATC GGG GAT GAC
91    Ile Ile Thr Thr Glu Glu Gly Phe Lys Thr Val Ile Gly Asp Asp

330   GAG TAT CTC CAC TTC CAC CAC CGG ATG CCA TCC TCT AAC GTG CGC
106   Glu Tyr Leu His Phe His His Arg Met Pro Ser Ser Asn Val Arg

375   TCA GTG GAG GTG GGC GGA GAC GTG CAG CTG CAT TCT GTG AAG ATC
121   Ser Val Glu Val Gly Gly Asp Val Gln Leu His Ser Val Lys Ile

420   TTC TGA GCAAGGACCC AGGGGCTTGG CGAGTGGGGG TGGGGTTTCG TCAGATCGTA
136   Phe Stop

476   GAGGAGGGTT GTGGATGGCG AATAAACTGT AGCTGTAGTTC C poly (A) -3'
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Rat      1  MSATHHKTPL PQGVRLGTVM RIRGVVPDQA GRFHVNLLCG EEQEADCALH
            ||   ||+ | |+|+| |||+ ||||+|| +|  ||||||||| ||| +| |||
Human    1  MSNVPHKSSL PEGIRPGTVL RIRGLVPPNA SRFHVNLLCG EEQGSDAALH

Rat     51  FNPRLDTSEV VFNTKQQGKW GREERGTGIP FQRGQPFEVL IITTEEGFKT
            |||||||||| |||+|+|| | |||||| |+| |||||||||| || +++|||
Human   51  FNPRLDTSEV VFNSKEQGSW GREERGPGVP FQRGQPFEVL IIASDDGFKA

Rat    101  VIGDDEYLHF HHRMPSSNVR SVEVGGDVQL HSVKIF
            |+|| +| ||  ||+| + ||  |||||||||  ||+||
Human  101  VVGDAQYHHF RHRLPLARVR LVEVGGDVQL DSVRIF
```

Figure 5

CLONE 4 **RAT MIA/CD-RAP**

5'-TT GAAGTCCATG

```
 13    ATG GTG TGC TCC CCA GTG CTC CTT GGT ATT GTC ATC TTG TCT GTT
  1    Met Val Cys Ser Pro Val Leu Leu Gly Ile Val Ile Leu Ser Val

 58    TTT TCA GGC CTC AGC AGG GCT GAT CGA GCC ATG CCC AAG CTG GCT
 16    Phe Ser Gly Leu Ser Arg Ala Asp Arg Ala Met Pro Lys Leu Ala

103    GAC CGG AAG CTG TGT GCA GAT GAG GAG TGT AGC CAT CCT ATC TCC
 31    Asp Arg Lys Leu Cys Ala Asp Glu Glu Cys Ser His Pro Ile Ser

148    ATG GCT GTG GCC CTT CAG GAC TAC GTG GCC CCT GAT TGC CGC TTC
 46    Met Ala Val Ala Leu Gln Asp Tyr Val Ala Pro Asp Cys Arg Phe

193    TTG ACT ATA TAC AGG GGC CAA GTG GTA TAT GTC TTC TCC AAG TTG
 61    Leu Thr Ile Tyr Arg Gly Gln Val Val Tyr Val Phe Ser Lys Leu

238    AAA GGC CGT GGA CGG CTT TTC TGG GGA GGC AGT GTG CAG GGA GAT
 76    Lys Gly Arg Gly Arg Leu Phe Trp Gly Gly Ser Val Gln Gly Asp

283    TAC TAT GGA GAC CTG GCA GCC CAC CTG GGC TAT TTC CCC AGT AGC
 91    Tyr Tyr Gly Asp Leu Ala Ala His Leu Gly Tyr Phe Pro Ser Ser

328    ATT GTC CGG GAG GAC CTG ACT CTG AAA CCT GGC AAA GTC GAT ATG
106    Ile Val Arg Glu Asp Leu Thr Leu Lys Pro Gly Lys Val Asp Met

373    AAG ACA GAT GAA TGG GAT TTC TAC TGT CAA TGA      GCTCAACCCA
121    Lys Thr Asp Glu Trp Asp Phe Tyr Cys Gln Stop

416    CCGATGTTAT CCCTGCAGTT ACCCTTCCGG TTTGGGCAAA TACAGCGGCC AACTGCAAAG
476    TGTTTTGTCC CTTTGGTTTT TGGGGTGGGC ATGTACAAAG AATGTTTCAC GGGTTCCTGA
536    ACCTAGCCAA TTAAAGCCCT GAATGTTGTA ACGTC poly (A)-3'
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Rat          MVCSPVLLGI  VILSVFSGLS RADRAMPKLA DRKLCADEEC SHPISMAVAL
Rat CD-RAP   (partial)                          LCADEEC SHPISVTVAL
Mouse MIA    MVWSPVLLGI  VVLSVFSGPS RADRAMPKLA DWKLCADEEC SHPISMAVAL
Cow CD-RAP   MAWSLVFLGV  VLLSAFPGPS AGGRPMPKLA DRKMCADEEC SHPISVAVAL
Human MIA    MARSLVCLGV IILLSAFSGPG VRGGPMPKLA DRKLCADQEC SHPISMAVAL

Rat           QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAAHLG
Rat CD-RAP    QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAAHLG
Mouse MIA     QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGG YYGDLAARLG
Cow CD-RAP    QDYVAPDCRF LTIHQGQVVY IFSKLKGRGR LFWGGSVQGD YYGDGAARLG
Human MIA     QDYMAPDCRF LTIHRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAARLG

Rat           YFPSSIVRED LTLKPGKVDM KTDEWDFYCQ
Rat CD-RAP    YFPSSIVRED LTLKPGKVDM KTDE
Mouse MIA     YFPSSIVRED LNSKPGKIDM KTDQWDFYCQ
Cow CD-RAP    YFPSSIVRED QTLKPAKTDV KTDIWDFYCQ
Human MIA     YFPSSIVRED QTLKPGKVDV KTDKWDFYCQ
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Figure 6

REPRINT 1

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The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet

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The objective of the present study was to investigate whether the anticarcinogenic activity of conjugated linoleic acid (CLA) is affected by the amount and composition of dietary fat consumed by the host. Because the anticancer agent of interest is a fatty acid, this approach may provide some insight into its mechanism of action, depending on the outcome of these fat feeding experiments. For the fat level experiment, a custom formulated fat blend was used that simulates the fatty acid composition of the US diet. This fat blend was present at 10, 13.3, 16.7 or 20% by weight in the diet. For the fat type experiment, a 20% (w/w) fat diet containing either corn oil (exclusively) or lard (predominantly) was used. Mammary cancer prevention by CLA was evaluated using the rat dimethylbenz[*a*]anthracene model. The results indicated that the magnitude of tumor inhibition by 1% CLA was not influenced by the level or type of fat in the diet. It should be noted that these fat diets varied markedly in their content of linoleate. Fatty acid analysis showed that CLA was incorporated predominantly in mammary tissue neutral lipids, while the increase in CLA in mammary tissue phospholipids was minimal. Furthermore, there was no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in the phospholipid fraction. Collectively these carcinogenesis and biochemical data suggest that the cancer preventive activity of CLA is unlikely to be mediated by interference with the metabolic cascade involved in converting linoleic acid to eicosanoids. The hypothesis that CLA might act as an antioxidant was also examined. Treatment with CLA resulted in lower levels of mammary tissue malondialdehyde (an end product of lipid peroxidation), but failed to change the levels of 8-hydroxydeoxyguanosine (a marker of oxidatively damaged DNA). Thus while CLA may have some antioxidant function *in vivo* in suppressing lipid peroxidation, its anticarcinogenic activity cannot be accounted for by protecting the target cell DNA against oxidative damage. The finding that the inhibitory effect of CLA maximized at 1% (regardless of the availability of linoleate in the diet) could conceivably point to a limiting step in the capacity to metabolize CLA to some active product(s) which is essential for cancer prevention.

Introduction

Conjugated linoleic acid (CLA*) is a positional and geometric isomer of linoleic acid (1). It is a minor fatty acid found preferentially in beef and dairy products (2). In contrast to linoleic acid, which has been found consistently to enhance mammary tumorigenesis in rodents over a wide concentration range (3–5), CLA expresses an inhibitory effect at levels of 1% or less in the diet (6,7). Recently, we described two distinct activities of CLA in mammary cancer prevention with the use of the methylnitrosourea (MNU) model (8). First, exposure to CLA during the early post-weaning and peripubertal period only (21–42 days of age) is sufficient to block subsequent tumorigenesis induced by a single dose of MNU given at 56 days of age. This observation suggests that CLA is able to effect certain changes in the immature mammary gland and render it less susceptible to neoplastic transformation later in life. Second, CLA is also active in suppressing tumor promotion/progression. However, this mode of action is different from the first in that once the mammary cells have been initiated by a carcinogen, a continuous intake of CLA is necessary to achieve maximal inhibition.

The above cited studies on CLA chemoprevention (6–8) were carried out in rats fed a 5% (w/w) fat diet formulated with corn oil. Currently, there is no information as to whether an increase in the level of fat or a substitution of the type of fat in the diet might affect the cancer inhibitory efficacy of CLA. The experiments described in this paper were designed to address this question. Because the anticancer agent of interest is a fatty acid, it is anticipated that the approach will provide some insight into its mechanism of action, depending on the outcome of these fat feeding experiments. For the fat level experiment, a custom formulated fat blend was used that simulates the fatty acid composition of the US diet. The idea was to examine the efficacy of CLA in the context of a fat consumption habit (10–20% by weight) that is relevant to humans. For the fat type experiment, a 20% (w/w) fat diet containing either corn oil (exclusively) or lard (predominantly) was used. Corn oil and lard differ significantly in their content of linoleate. Therefore, changes in the inhibitory activity of CLA in the presence of these two fat types may point to a possible interaction between CLA and linoleic acid in modulating tumor growth. Mammary cancer prevention by CLA under these various dietary conditions was evaluated using the rat dimethylbenz[*a*]anthracene (DMBA) model.

Previous work by Ha *et al.* (9) suggested that CLA is a potent antioxidant. At a molar ratio of 1 part CLA to 1000 parts linoleic acid, peroxide formation was reduced by >90% in a test tube assay. In fact, CLA was superior to tocopherol in this regard. In order to investigate whether interference with oxidative processes in cells might be implicated in cancer prevention by CLA, we examined the effect of CLA on two markers of cellular oxidative damage in the mammary tissue of rats fed either a high corn oil (unsaturated fat) or high lard (saturated fat) diet. These markers were malondialdehyde

*Abbreviations: CLA, conjugated linoleic acid; MNU, methylnitrosourea; DMBA, 7,12-dimethylbenz[*a*]anthracene; MDA, malondialdehyde; 8-OHdG, 8-hydroxydeoxyguanosine; TBA, thiobarbituric acid; AOS, antioxidant solution; dG, deoxyguanosine.

(MDA), an end product of lipid peroxidation, and 8-hydroxydeoxyguanosine (8-OHdG), an oxidized base isolated from DNA. Lipid peroxidation products have been implicated in mediating the formation of 8-OHdG in DNA (10). A recent publication from Thompson's laboratory has also reported that the number of 8-OHdG residues in mammary gland DNA increased in proportion to the degree of fatty acid unsaturation (as determined by iodine value) in the diet oils (11). More importantly, the rate of increase was sensitive to the presence or absence of nutritional levels of antioxidants such as vitamin E and selenium. Because of the above findings, we felt that these markers would be appropriate in assessing whether the antioxidant activity of CLA is manifest *in vivo*. Our goal was to investigate the possible relationship between the modulation of oxidative damage and the efficacy of cancer protection by CLA.

Materials and methods

Source and composition of CLA and other dietary fats

The method of CLA synthesis from >99% pure linoleic acid was detailed in our earlier publication (6). CLA was custom ordered from Nu-Chek Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-, *t*9,*c*11- and *t*10,*c*12-CLA, constituted ~90% of the total. From our experience over several years, we have found that there were minimal variations in isomer distribution from batch to batch.

A 'vegetable fat blend' was prepared by Kraft Foods Inc. at their Technology Center (Glenview, IL). This fat blend was designed specifically to simulate the fatty acid composition in the average US diet. It consisted of 39.5% soybean oil, 22% palm oil, 12.5% high oleic sunflower oil, 9% cottonseed oil, 8.5% coconut oil and 8.5% cocoa butter. The reason that plant oils were used exclusively was to minimize the CLA content of the fat blend. Gas chromatographic analysis showed the following composition: C8:0, 0.9%; C10:0, 0.7%; C12:0, 5.1%; C14:0, 2.3%; C16:0, 18.8%; C16:1, 0.2%; C18:0, 5.6%; C18:1, 31.8%; C18:2, 29.5%; C18:3, 3.4%; C20:0, 0.4%; C22:0, 0.3%; CLA, not detectable. The above 'vegetable fat blend' has a polyunsaturate/monounsaturate/saturate fatty acid ratio of 1:1:1, which provided a fatty acid profile similar to that found in the typical US diet.

Two other commercial fats were used in this study: Mazola brand corn oil was obtained from Best Foods (Somerset, NJ) and lard was purchased from Harlan Teklad (Madison, WI). Lard contains ~0.3 mg CLA/g fat.

Design of mammary cancer chemoprevention experiments

Pathogen-free female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC) and housed in an environmentally controlled room with a 12 h light/12 h dark cycle. Mammary tumors were induced by a single i.g. dose of 7.5 mg DMBA at 50 days of age. Animals were palpated weekly to determine the time of appearance and location of tumors. At necropsy the mammary glands were exposed for the detection of non-palpable or microscopic tumors. Only histologically confirmed adenocarcinomas were reported in the results. In general between 10 and 15% of the tumors found in all groups (with or without CLA) were fibroadenomas. Tumor incidences at the final time point were compared by χ^2 analysis and the total tumor yield between groups was compared by frequency distribution analysis as described previously (12).

The first experiment involved feeding rats a diet containing different levels of the 'vegetable fat blend' at 10, 13.3, 16.7 and 20% by weight, with or without 1% CLA. Thus there were a total of eight dietary treatment groups in this design. All diets, which were prepared according to the AIN-76 formulation (6), were started 1 week before DMBA and continued until sacrifice (23 weeks post-DMBA). Ip has previously described the method of nutrient adjustment for diets containing different levels of fat so that the intake of protein, vitamins, minerals and calories was similar among the different groups (13).

At necropsy, the uninvolved (non-tumor-bearing) mammary glands from selected groups were excised and immediately dropped into liquid nitrogen. Upon removal from storage at –80°C, the frozen samples were pulverized and total fat was extracted with chloroform/methanol. The separation of phospholipids and neutral lipids was achieved with the use of a Sep-Pak silica cartridge as described in our earlier publication (6). Gas chromatographic analysis of the fatty acid methyl esters (including CLA) was determined by the method reported previously by Chin *et al.* (2).

The second experiment involved feeding a diet containing either 20% corn oil or a mixture of 8% corn oil + 12% lard, both with or without 1% CLA. Lard was chosen over tallow because of the much lower CLA content in lard (4 mg CLA/g fat in tallow versus 0.3 mg CLA/g fat in lard). The 12% lard in the diet therefore contributed <4 mg CLA/100 g diet, an amount that was insignificant compared with the level of 1% CLA used in this experiment. It should be noted that the lard diet also contained 8% corn oil. The reason was the previous finding of a high linoleate requirement for mammary tumorigenesis in the DMBA model (3,14). Similar to the above protocol, the feeding of the corn oil or lard diet ± 1% CLA was started 1 week before DMBA and continued until sacrifice.

The third experiment involved feeding a 20% corn oil diet with either 0.5, 1 or 1.5% CLA. The purpose was to determine the dose–response characteristics with respect to CLA in the presence of a linoleate-rich diet and to compare the results obtained here with our previous study of CLA efficacy (also at 0.5, 1 or 1.5%) in rats fed a 5% corn oil diet (6). Corn oil consists of ~60% linoleate. Thus the 5 and 20% corn oil diets contain ~3 and 12 g linoleate/100 g diet respectively. The purpose was to find out whether a diet that was rich in linoleate would require more CLA to achieve a maximal inhibitory effect.

Determination of MDA and 8-OHdG in mammary tissue

A separate experiment was set up to evaluate the effect of CLA on markers of lipid peroxidation and cellular oxidative damage in the mammary gland. Rats were fed the same corn oil or lard diet with or without 1% CLA as described in the above section. However, they were not treated with DMBA and the feeding period only lasted 2 months. At necropsy, the abdominal inguinal mammary gland chains (glands 4–6) were excised and dropped immediately into liquid nitrogen.

Tissue MDA was quantified as its thiobarbituric acid (TBA) derivative with reverse phase HPLC and photometric absorbance detection at 535 nm based on an extensive modification of the method described by Draper and Hadley (15). Mammary gland samples were homogenized with a Polytron in water containing a 1% antioxidant solution (AOS; 0.3 M dipyridyl and 2% butylated hydroxyanisole in ethanol), 1 part mammary tissue to 9 parts water (w/v). The samples were centrifuged at 6500 *g* and the fat plugs were removed, followed by further homogenization to resuspend the pellet. Since optimal reaction conditions were found to vary with protein concentration, an amount of homogenate containing ~1.2 mg protein was prepared for hydrolysis. The homogenate was combined with 7.5 µl 5 N HCl, 7.5 µl AOS and enough water to bring the volume to 1.5 ml. The covered tubes were heated to 96°C for 3 h. They were cooled quickly in tap water and 30 µl/tube sodium tungstate (Na_2WO_4) was added to facilitate precipitation of protein. After centrifugation at 6500 *g* for 10 min, 1 ml supernatant was then transferred to a clean glass tube. An aliquot of 0.75 ml TBA solution (1.11% TBA in 74 mM KOH) was added to each tube, followed by heating for 90 min to form the MDA–TBA adduct. Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA–TBA adduct was separated using a 4.6×150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50 mM potassium phosphate buffer, pH 6.0, delivered at 1.5 ml/min. Photometric absorbance detection was at 535 nm. MDA was quantified by comparison of sample peak heights to those of the standard prepared from 1,1,3,3-tetramethoxypropane. The final results are expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (BioRad Protein Assay; BioRad Laboratories, Richmond, CA).

For the assay of 8-OHdG, the various procedures of DNA purification from the mammary gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and deoxyguanosine (dG) by HPLC and the quantitation of 8-OHdG with an electrochemical detector were described in detail in a recent publication from Thompson's laboratory (11). The only modification introduced here was the elimination of phenol from the DNA isolation procedure. Detector response was linear from 10 to >800 pg/injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues 8-OHdG/10^6 residues dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Results

Table I summarizes the mammary cancer chemoprevention data of CLA in rats fed different levels of fat. Tumor incidence at the time of necropsy was significantly reduced ($P < 0.05$) by CLA treatment in each of the four fat groups. In the absence of CLA supplementation, the total number of tumors increased by ~40% (from 71 to 98) in the range 10–20% dietary fat

Table I. Mammary cancer prevention by CLA in rats fed different levels of fat[a]

Dietary fat level	CLA	Tumor incidence	Total no. of tumors	Inhibition (%)[b]
10%		68.8%	71	
10%	1%	40.6%[c]	31[c]	56%
13.3%		81.3%	74	
13.3%	1%	46.9%[c]	40[c]	46%
16.7%		87.5%	94	
16.7%	1%	59.4%[c]	46[c]	51%
20%		90.6%	98	
20%	1%	59.4%[c]	49[c]	50%

[a]The fat used in this experiment was the 'vegetable fat blend' as described in Materials and methods. There were 32 rats/group.
[b]Percent inhibition was calculated using the tumor number data.
[c]$P < 0.05$ compared with the corresponding control group without CLA.

intake. However, as indicated in the last column of the table, the magnitude of tumor inhibition in CLA-treated rats was fairly consistent across all fat groups: 56% reduction on the 10% fat diet, 46% reduction on the 13.3% fat diet, 51% reduction on the 16.7% fat diet and 50% reduction on the 20% fat diet. This observation suggests that the efficacy of CLA in mammary cancer prevention is independent of the level of fat in the diet. The number of fibroadenomas found across all groups was very low and CLA did not affect the formation of these benign lesions.

The uninvolved (non-tumor-bearing) mammary glands of rats from selected groups were processed for fatty acid analysis in the neutral lipid and phospholipid fractions. The results from four dietary treatment groups (10 and 20% fat ± CLA) are presented in Table II. The data are expressed as percentages of total fatty acids. As indicated in footnote b, each value represents the mean of seven to eight samples, but since the standard error of the group mean is generally within 5% of the mean, the SEM is omitted from the table in order to make it more readable.

In the neutral lipid fraction, the three predominant fatty acids were C16:0, C18:1 and C18:2. In rats fed 10 and 20% fat without CLA the most significant change was an increase in C18:2 incorporation ($P < 0.05$) in the 20% fat group. The feeding of 1% CLA in diets containing 10 and 20% fat increased the netural lipid CLA content by 17.5-fold (from 0.2 to 3.5%) and 12-fold (from 0.2 to 2.4%) respectively, but did not alter the proportion of the other fatty acids in any substantial way.

Analysis of the phospholipid fraction showed that C16:0, C18:0, C18:2 and C20:4 accounted for >90% of total fatty acids. In particular, the high level of C20:4 incorporation was a distinctive characteristic of phospholipids. Thus the fatty acid profile found in phospholipids was different from that found in neutral lipids. Again there was no evidence that CLA supplementation perturbed the distribution of linoleate or other fatty acids in phospholipids. Interestingly, the increase in CLA incorporation in phospholipids (~0.3%) was much smaller in magnitude compared with that observed in neutral lipids (~2–3%). These findings suggest that there is selectivity of CLA incorporation in different classes of lipid.

Table III shows the mammary cancer chemopreventive activity of CLA in rats fed either an unsaturated fat (corn oil) or a saturated fat (lard) diet. It was apparent from the data that CLA was equally effective in suppressing tumorigenesis regardless of the type of dietary fat intake. Furthermore, the magnitude of tumor inhibition seen in this experiment was very similar to that described in the first experiment (Table I). In other words, with a constant dose of DMBA, feeding of 1% CLA reduced the number of mammary tumors by ~50% and this activity was evidently unaffected by the fat content (level or type) in the diet.

The efficacy of CLA in inhibiting lipid peroxidation and oxidative damage in mammary tissue was assessed by measuring MDA in mammary gland homogenate and 8-OHdG in mammary gland DNA. The results are presented in Table IV. In this experiment rats were fed the same corn oil or lard diet with or without 1% CLA as in the mammary carcinogenesis experiment shown in Table III. However, the animals were not treated with DMBA and they were sacrificed after 2 months of feeding. MDA levels were significantly elevated in rats fed the corn oil versus the lard diet ($P < 0.001$), this finding thus confirms the increased susceptibility of unsaturated fat to peroxidation. Feeding of CLA was associated with a reduction in MDA in the mammary tissue in both fat groups ($P < 0.001$). This effect was somewhat greater in rats fed a rich unsaturated fat diet (corn oil, 35% reduction; lard, 25% reduction; $P = 0.02$). Diet-associated differences in tissue levels of 8-OHdG were less remarkable. A 10–15% increase in 8-OHdG levels was detected with feeding the corn oil versus the lard diet ($P = 0.08$), however, tissue levels of this oxidized base were unaffected by CLA ($P = 0.42$).

It has been reported previously that 1% CLA produced a maximal inhibitory effect on mammary carcinogenesis in rats fed a 5% corn oil diet (6). No further protection was detected at levels above 1% CLA. In order to find out whether the dose–response characteristics with respect to CLA might be different in rats fed a 20% corn oil diet, an experiment was carried out to evaluate such a possibility (the protocol was otherwise identical to the previous 5% corn oil experiment with CLA supplemented at 0.5, 1 or 1.5%). As pointed out in Materials and methods, the difference in linoleate intake is substantial between a 5 and a 20% corn oil diet. If the action of CLA is totally dissociated from the availability of linoleic acid, the dose–response characteristics with respect to CLA are likely to be the same in rats consuming either a 5 or 20% corn oil diet. The results in Table V clearly show that maximal tumor inhibition was obtained with 1% CLA in rats fed a 20% corn oil diet. Increasing the concentration of CLA to 1.5% did not lead to a greater benefit in cancer protection.

Discussion

CLA is not the only fatty acid known to inhibit carcinogenesis. Eicosapentaenoic acid and docosahexaenoic acid, which are representative of the *n*-3 polyunsaturated fatty acids in fish oil, also fit this category (16). However, CLA differs from the fish oil fatty acids in two distinct aspects as far as their efficacies are concerned. Whereas fish oil is usually required at levels of ~10%, CLA at levels of 1% or less is sufficient to produce a significant cancer protective effect (7). Additionally, there are a number of papers which have indicated that an optimal ratio of fish oil to linoleate in the diet is critical in achieving maximal tumor inhibition (17–19). As can be seen from the present study, the potency of CLA in cancer prevention is largely dissociated from the quantity and type of dietary fats consumed by the host.

A possible mechanism of cancer prevention by fish oil *n*-3

Table II. CLA incorporation in neutral lipid and phospholipid fractions of mammary gland[a]

Fatty acid	Neutral lipid[b]				Phospholipid[b]			
	10% fat		20% fat		10% fat		20% fat	
	–CLA	+CLA	–CLA	+CLA	–CLA	+CLA	–CLA	+CLA
C12:0	1.2	1.2	1.6	1.6				
C14:0	1.7	1.9	1.7	1.6	1.1	1.1	1.2	1.1
C16:0	24.3	24.7	21.3	20.5	19.6	19.5	20.4	21.1
C16:1	3.9	3.9	3.0	1.5	0.5	0.4	0.4	0.3
C18:0	3.9	3.6	3.7	4.8	37.3	38.1	38.2	37.6
C18:1	42.3	38.9	40.4	38.8	5.1	4.9	4.6	4.8
C18:2	20.9	21.0	26.2	27.0	11.5	11.3	10.7	11.6
C18:3	0.9	0.9	1.3	1.2	0.5	0.4	0.4	0.3
C20:4	0.7	0.4	0.6	0.6	24.3	23.9	23.3	23.2
CLA	0.2	3.5	0.2	2.4	0.1	0.4	0.1	0.4
	100	100	100	100	100	100	100	100

[a]The samples were processed from uninvolved glands of rats reported in Table I.
[b]Results are expressed as percent of total fatty acids. The sum of each column is equal to 100%. Each value represents the mean of 7–8 samples, the SEM generally being within 5% of the mean.

Table III. Mammary cancer prevention by CLA in rats fed either an unsaturated fat or a saturated fat diet[a]

Dietary fat	CLA	Tumor incidence	Total no. of tumors	Inhibition (%)[b]
Corn oil		83.3%	68	
Corn oil	1%	40.0%[c]	35[c]	49%
Lard		80.0%	60	
Lard	1%	40.0%[c]	32[c]	47%

[a]The unsaturated fat diet contained 20% corn oil, while the saturated fat diet contained 8% corn oil + 12% lard. There were 30 rats per group.
[b]Percent inhibition was calculated using the tumor number data.
[c]$P < 0.05$ compared with the corresponding control group without CLA.

Table IV. Effect of CLA feeding on MDA and 8-OHdG levels in mammary gland[a,b]

Dietary fat	Malondialdehyde (nmol/mg protein)[c]		8-OHdG (residues/10^6 dG)[d]	
	–CLA	+CLA	–CLA	+CLA
Corn oil	1.39 ± 0.08	0.90 ± 0.14	4.00 ± 0.26	4.05 ± 0.20
Lard	0.43 ± 0.03	0.32 ± 0.02	3.38 ± 0.26	3.75 ± 0.30

[a]Rats were fed either the corn oil or lard diet with or without 1% CLA for 2 months.
[b]Results are expressed as mean ± SE ($n = 9$).
[c]By factorial analyses of variance the following effects on malondialdehyde were noted. Type of fat, F ratio 88.903, $P < 0.001$; CLA, F ratio 13.76, $P = 0.001$; interaction between fat type and CLA, F ratio 5.62, $P = 0.024$.
[d]By factorial analyses of variance the following effects on 8-OHdG were noted. Type of fat, F ratio 3.18, $P = 0.08$; CLA, F ratio 0.42, $P = 0.42$; interaction between fat type and CLA, F ratio 0.37, $P = 0.54$.

polyunsaturated fatty acids has been postulated to be through perturbation of eicosanoid biosynthesis (19,20). *In vivo*, linoleic acid is converted to arachidonic acid, which is the precursor for the various eicosanoids produced via either the cyclo-oxygenase or lipoxygenase pathways. The data presented in this paper tend to suggest that CLA is unlikely to interfere with the metabolic cascade involved in converting linoleic acid to eicosanoids. First, the anticarcinogenic efficacy of CLA was not affected by variations in linoleate intake, as demonstrated by the experiments reported in Tables I and III.

Table V. Mammary cancer prevention by different levels of CLA in rats fed a 20% corn oil diet[a]

CLA	Tumor incidence	Total no. of tumors	Inhibition (%)[b]
	93.3%	87	
0.5%	70.0%	53[c]	39%
1%	50.0%[c]	37[c]	57%
1.5%	46.7%[c]	34[c]	61%

[a]There were 30 rats per group.
[b]Percent inhibition was calculated using the tumor number data.
[c]$P < 0.05$ compared with the control group without CLA.

Second, similar dose–response characteristics with respect to CLA at 1% and below were noted in rats fed either a 5 or 20% corn oil diet (6; Table V). No further protection was evident with supplementation of CLA above 1% in both cases. The fact that the effect of CLA maximizes at 1% may indicate a limiting step in the capacity to metabolize CLA to some active product(s) which is essential for inhibition of carcinogenesis. Suffice it to note that absorption of CLA is probably not a confounding factor here, because tissue accumulation of CLA continues to rise with dietary levels above 1% (unpublished data).

In all the carcinogenesis experiments included in this paper, CLA was given to the animals starting 1 week before DMBA and continuing until termination of the experiment. We adopted this protocol initially with the experiment shown in Table I, and in order to maintain uniformity, followed the same protocol in subsequent experiments reported in Tables III and V. However, we have observed that CLA does not affect DMBA binding to mammary cell DNA (7) nor does it affect phase II conjugating enzymes, such as glutathione S-transferase and UDP-glucuronyl transferase (6). In other words, CLA is expected to have little influence on DMBA activation or detoxification. It can thus be conjectured that the major impact of CLA on mammary carcinogenesis with the above protocol is due to its inhibitory effect on tumor promotion or progression.

Some explanation is called for here about the finding that in rats which were maintained on the 'vegetable fat blend' diet there was a small but detectable amount of CLA in the mammary tissue even though the animals did not receive an

exogenous supply of CLA. In an attempt to determine whether the bacterial flora in the colon of rats could be the source of CLA, Chin *et al.* (21) have recently examined the tissue levels of CLA between conventional and germ-free rats which were fed diets with or without free linoleic acid. With the conventional rats, tissue CLA concentrations were 5–10 times higher in those animals given a 5% linoleic acid supplement. In contrast, CLA concentrations in tissues of germ-free rats were not affected by the addition of linoleic acid. These findings strongly suggest that the intestinal bacterial flora of rats is capable of converting linoleic acid to CLA.

As shown by the data in Table II, there might be some selectivity in the incorporation of CLA into different lipids following ingestion of a diet rich in CLA. When expressed as a percentage of total fatty acids, CLA is more abundant in neutral lipids than in phospholipids. It is unclear whether this uneven distribution of CLA in various lipid fractions has any relevance to cancer risk modulation. Because of the configuration of the *trans* double bond(s) in CLA, the incorporation of CLA in membrane phospholipids could conceivably diminish the fluidity of the lipid bilayer. On the other hand, the small amount of CLA in phospholipids tends to argue against the significance of a membrane effect. The storage of CLA in neutral lipids could portend the importance of this pool in providing a continuous supply of CLA for generation of some active metabolite(s). Further research is needed to examine the rate of turnover of CLA in neutral lipids and the possible oxidative modification of CLA, similar to that observed with linoleic acid (22–24).

The ability of CLA to suppress lipid peroxidation was first described by Pariza's laboratory (9). In that work linoleic acid was exposed to air and moderate heat with or without a very small amount of CLA for an extended period of time. Under those conditions the degree of linoleic acid oxidation (peroxide value) was determined by the thiocyanate method (25). It was hypothesized that an oxidized derivative of CLA might be the active antioxidant species, rather than CLA itself (9). According to the proposed scheme, which is supported by spectrophotometric evidence, a β-hydroxy acrolein moiety would be introduced across the conjugated double bond of CLA following reaction with a hydroxyl or peroxyl radical and molecular oxygen. Antioxidant activity would result from chelation of iron by the β-hydroxy acrolein functional group, thereby interfering with the Fenton reaction. A recent paper by van den Berg (26), however, contradicted the above conclusion. These investigators studied whether CLA could protect membrane vesicles composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine from oxidative modification under various conditions. Oxidation was determined by direct spectrophotometric measurement of conjugated diene formation and by gas chromatographic/mass spectrometric analysis of fatty acids. It was found that CLA neither acts as a radical scavenger nor is it converted into a metal chelator in the Fe^{2+} ion-dependent oxidative reaction. Thus, at least in a model membrane system, CLA does not function as an effective antioxidant or antioxidant precursor.

The results presented in Table IV may provide new clues as to the effect of CLA on oxidative events *in vivo*. MDA levels were lower in mammary tissue of CLA-treated rats and the suppressive effect was somewhat greater in rats fed the more unsaturated dietary fat. Since MDA was measured in whole mammary gland homogenate, it is likely to represent the peroxidation of neutral lipids, which are found predominantly in the mammary gland adipocytes. As shown in Table II, CLA is also preferentially incorporated in the neutral lipid fraction. On the other hand, the levels of 8-OHdG, which are only marginally affected by the type of dietary fat and not at all by CLA supplementation, are probably a better indicator of DNA oxidative damage that may be causally related to tumor promotion/progression. The presence of 8-OHdG has been implicated in mismatching errors and base substitutions in DNA replication (27,28). The absence of a detectable effect of CLA on 8-OHdG is also consistent with the lack of a significant accumulation of CLA in the phospholipid fraction, which is likely to originate from mammary epithelial cells. In summary, based on the information obtained in this study, we believe that the ability of CLA to inhibit mammary carcinogenesis is not mediated by protecting the target cell DNA against damage induced by reactive oxygen species. Current research is focused on using a mammary epithelial cell culture model (29,30) to generate new insights into potential mechanisms of CLA in regulating growth and differentiation.

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